



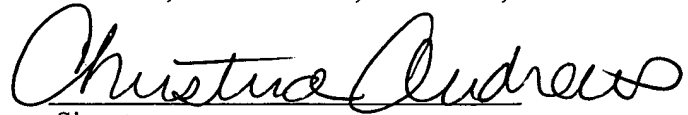
DOCKET NO: T0541.70000US06

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Richard F. Selden
Serial No: 08/465,596
Confirmation No. 2132
Filed: June 5, 1995
For: TRANSKARYOTIC IMPLANTATION
Examiner: Deborah Crouch, Ph.D.
Art Unit: 1632

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the 14th day of August, 2004.


Signature

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

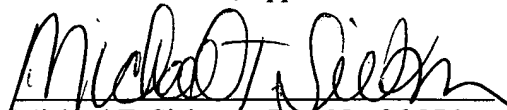
Transmitted herewith are the following documents:

- ☒ Request for Withdrawal of Notice of Abandonment
- ☒ Copy as Filed of Transmittal Letter dated June 21, 2004
- ☒ Copy as Filed of Amendment dated June 21, 2004
- ☒ Copy as Filed of Petition for 3 Month Extension of Time dated June 21, 2004
- ☒ Copy as Filed of References (Exhibits 1-5)
- ☒ Copy as Filed of Check #440019963 in the amount of \$950.00 marked with a notation check cashed by the USPTO on July 2, 2004
- ☒ Copy as Filed of Return Receipt Postcard date-stamped by the USPTO
- ☒ Return Receipt Postcard

If the enclosed papers are considered incomplete, the Mail Room and/or the Application Branch is respectfully requested to contact the undersigned at (617) 646-8000, Boston, Massachusetts.

No fee is required. If the fee is insufficient, the balance may be charged to Deposit Account 23/2825. A duplicate of this sheet is enclosed.

Respectfully submitted,
Richard F. Selden, Applicant


Michael T. Siekman, Reg. No. 36,276
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2211
Telephone: (617) 646-8000

Docket No. T0541.70000US06
Date: August 14, 2004
XNDD

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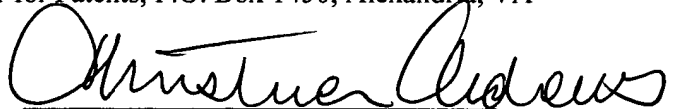
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Art Unit: 1632

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The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the 11th day of August, 2004.


Signature

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:


REQUEST FOR WITHDRAWAL OF NOTICE OF ABANDONMENT

On June 28, 2004, a Notice of Abandonment [Paper No. 200] was received by the undersigned attorney. Applicant respectfully requests that this Notice of Abandonment be withdrawn.

The Notice of Abandonment indicates a response was not received in response to the office action mailed on December 19, 2003. However, Applicant filed an Amendment on June 21, 2004, along with a Petition for Three Month Extension of Time. The extension of time fee of \$950.00 was paid to extend the time in which the amendment was due. Applicant files herewith a copy of the date-stamped postcard evidencing receipt of these papers by the USPTO. See M.P.E.P. § 505 at p. 500-16, col. 2.

Applicant respectfully requests written confirmation of the withdrawal of abandonment of this application by the Patent and Trademark Office.

Respectfully submitted,
Richard F. Selden, Applicant


Michael T. Siekman, Reg. No. 36,276
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2211
Telephone: (617) 646-8000

Docket No. T0541.70000US06
Date: August 11, 2004
XNDD



Serial No. 08/465,596 File No. 10541.70000US06 By: MTS

Title: TRANSKARYOTIC IMPLANTATION

Application of Richard F Selden WGS Date: 6/21/04

The U.S. PTO Mail Room acknowledges receipt of the following on the date stamped hereon:

<input type="checkbox"/> Mailing by Express Mail (37 CFR 1.6)	<input type="checkbox"/> Provisional Application Cover Sheet
<input type="checkbox"/> Express Mail Label No. _____	<input type="checkbox"/> Multiple Dependent Claim Fee Sheet
<input type="checkbox"/> Patent Application	<input type="checkbox"/> Inf. Disc. Statement, PTO Form 1449
<input type="checkbox"/> Non-provisional <input type="checkbox"/> Provisional	<input type="checkbox"/> References Cited
<input type="checkbox"/> Incl. _____ pages, (_____ pgs) Specification, # claim(s) _____	<input type="checkbox"/> Priority Document(s) # _____
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<input type="checkbox"/> Declaration(s) _____	<input checked="" type="checkbox"/> Amendment/Response
<input type="checkbox"/> Drawings _____ Sheet(s) (FIGS. _____)	<input checked="" type="checkbox"/> Petition for Ext. of Time (x2) <u>3mths</u>
<input type="checkbox"/> <input type="checkbox"/> Formal <input type="checkbox"/> Informal	<input type="checkbox"/> Issue Fee Transmittal
<input type="checkbox"/> Utility Patent Application Transmittal	<input type="checkbox"/> Assignment and Coversheet
<input type="checkbox"/> Fee calculation sheet (x2)	<input type="checkbox"/> Notice of Appeal
<input type="checkbox"/> CPA Transmittal	<input checked="" type="checkbox"/> Brief (x3)
<input type="checkbox"/> Verified Statement claiming small entity status	<input checked="" type="checkbox"/> Check for \$ <u>950.00</u> Check # <u>963</u>
<input type="checkbox"/> Request for Approval and Entry of Formal Drawings	<input checked="" type="checkbox"/> Transmittal Letter (x2)
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☒ References (Exhibits 1-5)

☐ Other _____

DATE MAILED June 21, 2004

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Wolf, Greenfield & Sacks, P.C.

PTM Account
600 Atlantic Avenue
Boston, MA 02210



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June 21, 2004

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THE Commissioner of Patents & Trademarks
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Pattina D. O'Brien
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10541/70000US06

⑈0019963⑈ ⑆211070175⑆ 1108202451⑈

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COPY AS FILED

Serial No. 08/465,596 File No. T0541.70000US06 By: MTS

Title: TRANSKARYOTIC IMPLANTATION

Application of Richard F Selden WGS Date: 6/21/04

The U.S. PTO Mail Room acknowledges receipt of the following on the date stamped hereon:

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| <input type="checkbox"/> Mailing by Express Mail (37 CFR 1.10) | <input type="checkbox"/> Provisional Application Cover Sheet |
| <input type="checkbox"/> Express Mail Label No. _____ | <input type="checkbox"/> Multiple Dependent Claim Fee Sheet |
| <input type="checkbox"/> Patent Application | <input type="checkbox"/> Inf. Discl. Statement, PTO Form 1449 |
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| Incl. _____ pages, (_____ pgs) Specification, | <input type="checkbox"/> Priority Document(s) # _____ |
| (_____ pgs) Abstract, (_____ pgs) Claims (_____ # claims) | <input type="checkbox"/> Copy of Notice to File Missing Parts |
| <input type="checkbox"/> Design Patent Application | <input checked="" type="checkbox"/> Amendment/Response |
| <input type="checkbox"/> Declaration(s) _____ | <input checked="" type="checkbox"/> Petition for Ext. of Time (x2) <u>3 months</u> |
| <input type="checkbox"/> Drawings _____ Sheet(s) (FIGS. _____) | <input type="checkbox"/> Issue Fee Transmittal |
| <input type="checkbox"/> Formal <input type="checkbox"/> Informal | <input type="checkbox"/> Assignment and Coversheet |
| <input type="checkbox"/> Utility Patent Application Transmittal | <input type="checkbox"/> Notice of Appeal |
| <input type="checkbox"/> Fee calculation sheet (x2) | <input type="checkbox"/> Brief (x3) |
| <input type="checkbox"/> CPA Transmittal | <input checked="" type="checkbox"/> Check for \$ <u>950.00</u> Check # <u>963</u> |
| <input type="checkbox"/> Verified Statement claiming small entity status | <input checked="" type="checkbox"/> Transmittal Letter (x2) |
| <input type="checkbox"/> Request for Approval and Entry of Formal Drawings | <input checked="" type="checkbox"/> Cert. of Mailing under 37 CFR 1.8(a) |
| <input checked="" type="checkbox"/> Other <u>References (Exhibits 1-5)</u> | |

DATE MAILED June 21, 2004



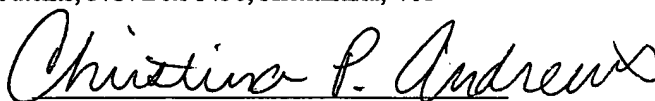
DOCKET NO: T0541.70000US06

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner: Deborah Crouch, Ph.D.
Art Unit: 1632

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the 21st day of June, 2004.


Christina P. Andrews

Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

COPY AS FILED

Sir:

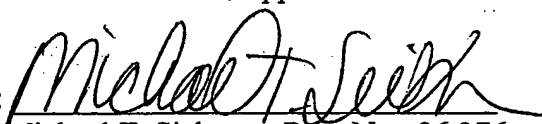
Transmitted herewith are the following documents:

- ☒ Amendment
- ☒ Petition for 3 Month Extension of Time
- ☒ References (Exhibits 1-5)
- ☒ Return Receipt Postcard

If the enclosed papers are considered incomplete, the Mail Room and/or the Application Branch is respectfully requested to contact the undersigned at (617) 720-3500, Boston, Massachusetts.

A check in the amount of \$950.00 is enclosed to cover the filing fee. If the fee is insufficient, the balance may be charged to Deposit Account 23/2825. A duplicate of this sheet is enclosed.

Respectfully submitted,
Richard F Selden, Applicant

By: 
Michael T. Siekman, Reg. No.: 36,276
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2211
Telephone: (617)720-3500

Docket No. T0541.70000US06
Date: June 21, 2004
xJune 21, 2004x



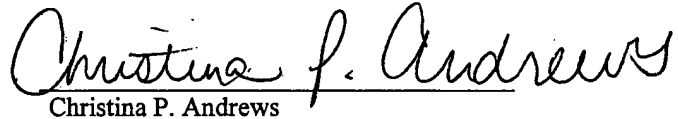
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Christina P. Andrews

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

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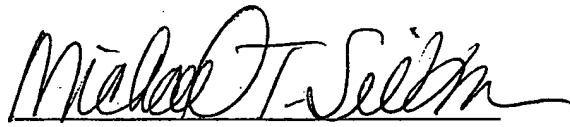
PETITION FOR THREE MONTH EXTENSION OF TIME

Sir:

A three (3) month extension of time, to and including June 21, 2004, is requested for response to the Patent Office Communication of December 19, 2003.

The extension fee of \$950.00 as set forth in 37 C.F.R. §1.17(a) is enclosed herewith. If the amount is insufficient, the balance may be charged to Deposit Account No. 23/2825.

Respectfully submitted,
Richard F Selden, Applicant


Michael T. Siekman, Reg. No. 36,276
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600 Atlantic Avenue
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Telephone: (617) 720-3500

Docket No. T0541.70000US06
Date: June 21, 2004
x06/21/2004
803020.1



DOCKET NO: T0541.700000US06

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Richard F Selden
Serial No: 08/465,596
Filed: June 5, 1995
For: TRANSKARYOTIC IMPLANTATION
Examiner: Deborah Crouch, Ph.D.
Art Unit: 1632

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Christina P. Andrews
Christina P. Andrews

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

COPY AS FILED

AMENDMENT

Sir:

In response to the Office Action mailed December 19, 2003 [Paper No. 20], please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims that begins on page 2 of this amendment.

Remarks begin on page 6 of this amendment.

In the Claims

Applicant has submitted a new complete claim set showing marked up claims with insertions indicated by underlining and deletions indicated by strikeouts and/or double bracketing.

Please amend pending claims 72-73, 84, and 104 as noted below.

~~Claims 1-71 (cancelled).~~

72 (currently amended): A method of transferring a gene into a recipient subject, comprising:

- (a) transfecting immortalized somatic cells *in vitro* with a DNA sequence by chemical or physical techniques to introduce the DNA sequence into the cells;
- (b) screening the resulting transfected immortalized somatic cells *in vitro* to select a cell, wherein the selected cell is stably transfected with the DNA sequence so that the selected cell has the permanent capacity to direct expression of the DNA sequence;
- (c) cloning and expanding the selected immortalized somatic cell *in vitro*; and
- (d) injecting the resulting transfected, screened, cloned, and expanded immortalized somatic cells into the recipient subject;

wherein the DNA sequence comprises the gene and a promoter capable of functioning in the immortalized somatic cells; and

wherein, following injection of the transfected, screened, cloned, and expanded immortalized somatic cells into the recipient subject, the DNA sequence is incapable of recombining with endogenous retroviral sequences, and the DNA sequence is incapable of initiating chronic viral infection in the recipient subject.

73 (currently amended). The method of claim 72, wherein the immortalized somatic cells are human cells.

74 (previously presented). The method of claim 73, wherein the human cells are selected from the group consisting of fibroblasts, myocytes, hepatocytes, kidney capsular cells, endothelial cells, epithelial cells of the gut, and pituitary cells.

75 (previously presented). The method of claim 73, wherein the gene encodes a hormone, an enzyme, or a receptor.

76 (previously presented). The method of claim 73, wherein the gene encodes human growth hormone.

77 (previously presented). The method of claim 73, wherein the gene encodes human insulin.

78 (previously presented). The method of claim 73, wherein the transfection comprises calcium phosphate-mediated transfection, microinjection, electroporation, or DEAE-dextran transfection.

79-81 (cancelled).

82 (previously presented). The method of claim 73, wherein the promoter is a regulatable promoter.

83 (previously presented). The method of claim 73, wherein the DNA sequence further comprises a selectable gene, and wherein the promoter is operably linked to the selectable gene.

84 (currently amended). The method of claim 73, wherein the screening step further comprises screening the resulting transfected immortalized somatic cells *in vitro* to select a cell possessing desired expression properties.

85-103 (cancelled).

104 (currently amended). A method of transferring a gene into a recipient subject, comprising:

- (a) providing immortalized somatic cells;
- (b) transfecting the immortalized somatic cells *in vitro* with a DNA sequence comprising the gene and a promoter capable of functioning in the immortalized somatic cells, wherein the gene encodes a gene product, and wherein the immortalized somatic cells are stably transfected with the gene so that the immortalized somatic cells have the permanent capacity to direct expression of the gene upon induction of the promoter;
- (c) screening the resulting transfected immortalized somatic cells *in vitro* to select a transfected immortalized somatic cell, wherein the screening comprises characterizing the transfected immortalized somatic cell with respect to expression and regulation of the gene by assaying for translation of the mRNA into the gene product;
- (d) cloning and expanding, *in vitro*, the transfected and screened immortalized somatic cell selected in step (c) to form 10^5 - 10^{10} transfected, screened, cloned, and expanded immortalized somatic cells, and
- (e) combining the 10^5 - 10^{10} transfected, screened, cloned, and expanded immortalized somatic cells with a physiologically acceptable buffer or carrier; and
- (f) injecting the resulting transfected, screened, cloned and expanded cell preparation into the recipient subject,

wherein, following injection of the transfected, screened, cloned, and expanded immortalized somatic cells into the recipient subject, the DNA sequence is incapable of recombining with endogenous retroviral sequences, and the DNA sequence is incapable of initiating chronic viral infection in the recipient subject.

105 (previously presented). The method of transferring a gene into a recipient subject of any one of claims 73 or 104, wherein the transfected gene encodes human growth hormone.

106 (previously presented). The method of transferring a gene into a recipient subject of any one of claims 73 or 104, wherein the transfected gene encodes insulin.

107 (previously presented). The method of transferring a gene into a recipient subject of any one of claims 73 or 104, wherein the DNA sequence integrates into the chromosome of the selected cell.

108 (previously presented). The method of transferring a gene into a recipient subject of any one of claims 73 or 104, wherein the DNA sequence replicates as an extrachromosomal plasmid.

REMARKS

Claims 72-78, 82-84, and 104-108 were previously pending in this application. Claims 72-73, 84, and 104 have been amended. As a result, claims 72-78, 82-84, and 104-108 are pending for examination, with claims 72 and 104 being independent claims.

Claims 72-73 84, and 104 have been amended to recite that the transfected immortalized somatic cells are "immortalized" somatic cells. The application supports this amendment at, *inter alia*, page 37, lines 27-29, where the transfected cells used in the examples are distinguished from "primary cells." Those transfected cells used in the examples are "[c]ultured mouse Ltk fibroblasts"—an immortalized cell line. [Page 40, lines 5-7.] Thus, no new matter has been added.

Provisional Obviousness-type Double Patenting
Rejection Over Claims of Application Serial No. 08/461,292

The Examiner provisionally rejected all the pending claims over the pending claims of co-filed Application Serial No. 08/461,292. [Paper No. 20 at p. 2.] Applicant intends to file a terminal disclaimer over Application Serial No. 08/461,292 upon withdrawal of the remaining rejections. Because this is a provisional rejection, Applicant need not address it further at this time.

Provisional and Actual Obviousness-type Double
Patenting Rejection Over Claims of Later-filed Applications

The Examiner provisionally and actually rejected all the pending claims over certain claims from later-filed applications and/or the resulting patents, specifically, Application Serial No. 09/549,200, Patent No. 6,303,379, Patent No. 6,048,729, and Patent No. 6,054,288. [Paper No. 20 at pp. 2-13.] Applicant notes that, as the Examiner indicates [*e.g.*, page 4, lines 12-13; page 6, line 11; page 9, lines 4-5; page 11, lines 13-17], the claims of these later filed applications and the resulting patents specifically recite that the transfected cells are either primary cells or secondary cells. Applicant has amended all the pending claims to recite that the transfected immortalized somatic cells are immortalized somatic cells.

The Examiner has conceded that two-way obviousness is required to support the double patenting rejections over the later-filed applications and resulting patents. [Paper No. 20 at p. 13,

lines 9-12.] Thus, these rejections must be withdrawn if either the pending claims are nonobvious over the claims of the later-filed applications and resulting patents, or the claims of the later-filed applications and resulting patents are nonobvious over the pending claims. "If **either** analysis does not compel a conclusion of nonobviousness, no double patenting rejection of the obvious-type is made" M.P.E.P. § 804 at p. 800-23, col. 2 (emphasis added).

The double patenting rejections should be withdrawn because the later-filed primary or secondary cell claims would not have been obvious over the earlier-filed immortalized cell claims pending in this application. One of ordinary skill in the art familiar with such gene transfer to a recipient subject with immortalized cells would not have been motivated to attempt it with primary or secondary cells. Moreover, even if such motivation did exist, there would have been no reasonable expectation of success. Thus, these provisional and actual obviousness-type double patenting rejections should be withdrawn.

One of ordinary skill in the art familiar with such immortalized cell work would not have been motivated to attempt it with primary or secondary cells because primary and secondary immortalized somatic cells are very different from immortalized cells. These differences would have made primary and secondary cells less desirable to one of ordinary skill in the art than immortalized cells. For example, primary and secondary cells have finite life spans in culture, where growth generally slows and arrests after about 50 to 90 mean population doublings. In addition, over time, primary and secondary immortalized somatic cells generally exhibit an increase in cell doubling time and cell volume, and a decrease in saturation density and senescence. In contrast, immortalized cells in culture are generally characterized by a shorter doubling time, decreased cell volume, and increased saturation density. It is critical to note that immortalized cells exhibit an essentially unlimited capacity to divide *in vitro*; that is, they do not senesce. Because these characteristics would have made immortalized cells more desirable than primary or secondary cells for transferring a gene to a recipient subject, one of ordinary skill in the art would not have been motivated to substitute primary or secondary cells for immortalized cells.

Even if one of ordinary skill in the art would have been motivated to substitute primary or secondary cells for immortalized cells, one of ordinary skill in the art would not have had a reasonable expectation of success because primary and secondary cells handle DNA in a

fundamentally different manner than immortalized cells handle DNA. Immortalized cells are more susceptible to mutation and have a decreased ability to repair DNA. [Tsuji-mura et al., *Proc. Natl. Acad. Sci. USA* 87:1566-1570, 1990 (copy enclosed); McGregor et al., *Immortalized Somatic Cell and Molecular Genetics* 17:463-469, 1991; (copy enclosed).] Also, while telomeres shorten during the aging of a normal diploid cell, telomeres remain the same length in immortalized cells. [Harley et al., *Nature* 345:458-460, 1990 (copy enclosed).] In fact, immortalized cells possess a novel enzyme, telomerase, that prevents telomere shortening by adding DNA to the ends of chromosomes. Thus, the cellular processing of DNA in primary and secondary cells is fundamentally different from that in immortalized cells.

Holliday [TIG 5(2):42-45, 1989 (copy enclosed)] presents evidence of the differences between primary and secondary cells on the one hand and immortalized cells on the other hand. For example, it teaches that immortalized cells and primary and secondary cells handle DNA in dramatically different ways, with the genome of normal cells being much more stable than that of immortalized cells. For example, Holliday discusses differences in karyotype and the frequencies of non-disjunction of chromosomes, chromosome rearrangements, gene amplification, DNA methylation, and integration of foreign DNA between immortalized and primary or secondary immortalized somatic cells. Regarding integration of foreign DNA, Holliday states, at Page 44, Table 1, footnote d:

Several laboratories have failed to obtain stable transfectants with DNA integrated into a chromosome. (For obvious reasons, these negative results remain unpublished.) An illustration of this comes from a comparison of human diploid fibroblasts, strain MRC-5, and its SV40-transformed derivative. Both cells take up exogenous DNA, but stable transfection is very rare in the diploid parent, whereas it is frequent in the transformed derivative (L.I. Huschtscha, pers. commun.) However, DNA can be integrated in the chromosomes of eggs or embryonic cells, and into immortalized somatic cells using retrovirus vectors.

Chromosome non-disjunction and rearrangement events in immortalized cells, which are generally tumorigenic, and primary cells are compared at page 42 by Holliday as well:

Many tumour cell lines are heteroploid with a continually varying chromosome number. In such cells, there is probably at least one abnormal chromosome segregation per division, and there may be several. The overall frequency of nondisjunction is likely to be at least 100-fold higher than that of normal diploid cells. Some transformed or tumour

cells have been reported to have diploid, or quasi-diploid karyotypes, but such populations tend to give rise to hypo- or hyper-diploid cells. The pseudo-diploid Chinese hamster ovary (CHO) cell line has been used extensively in immortalized somatic cell genetics, and appropriate markers make it possible to measure chromosome nondisjunction. In hybrids heterozygous for two X chromosome-linked markers, abnormal segregation occurred at rates of $1.4 - 3.0 \times 10^{-3}$ per cell division in different experiments, which is almost certainly considerably higher than the rate in diploid cells.

While transformed, immortalized cells experience a high frequency of chromosome nondisjunction, normal diploid cells experience a low frequency. [Holliday, Table 1.] These events demonstrate the substantial differences in the abilities of non-immortalized and immortalized cells to repair DNA damage and to maintain a stable genome.

As exemplified by these fundamental genetic differences between immortalized and primary or secondary immortalized somatic cells, Holliday clearly shows that the genomes of normal cells are more stable than those of immortalized cells. Holliday shows that, at the time the presently claimed invention was made, immortalized cells were known to possess a "plastic" genetic structure that is easily manipulated. In contrast, primary and secondary cells simply were not expected to process DNA in the same manner as immortalized cells, and thus would not have been expected to be capable of undergoing stable transfection based on observations of immortalized cells.

An article by Mes-Mason [*J. Cell Sci.* 94:517-525, 1989 (copy enclosed)] even more clearly illustrates that one of ordinary skill in the art would not have had a reasonable expectation of success in substituting primary or secondary cells for immortalized cells to transfer a gene to a recipient subject. Like the Holliday article, the Mes-Mason article reports a failure in obtaining stably transfected primary cells. More importantly, the Mes-Mason article reports that this failure was remedied by immortalizing the primary cells. Thus, "no cell lines could be established after transfection of primary cells with pMTONCO DNA alone," but "[g]reater than 50% of primary cells isolated after transfection with the pSV2NEOSVEB1a plasmid could sustain growth in culture." [page 522, columns 1-2.] Thus, Mes-Mason was not able to isolate cell lines derived from stably transfected cells until those cells were immortalized with polyomavirus.

Accordingly, one of ordinary skill in the art would not have been motivated to substitute primary or secondary cells for immortalized cells to transfer a gene to a recipient subject. Even if such a motivation would have existed, one of ordinary skill in the art would not have had a reasonable expectation of success in substituting primary or secondary cells for immortalized cells. The obviousness-type double patenting rejections over the later-filed applications and resulting patents should, therefore, be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, this application should now be in condition for allowance. A notice to this effect is respectfully requested. If the Examiner believes, after this amendment, that the application is not in condition for allowance, the Examiner is requested to call the Applicant's attorney at the telephone number listed below.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Respectfully submitted,
Richard F Selden, Applicant

By: 

Michael T. Siekman, Reg. No. 36,276
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
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Telephone: (617) 720-3500

Docket No. T0541.700000US06
Date: June 21, 2004
June 21, 2004

Frequency of intrachromosomal homologous recombination induced by UV radiation in normally repairing and excision repair-deficient human cells

(xeroderma pigmentosum/gene conversion/rate of spontaneous recombination/hygromycin resistance)

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Communicated by Philip C. Hanawalt, December 4, 1989 (received for review August 14, 1989)

ABSTRACT To investigate the role of DNA damage and nucleotide excision repair in intrachromosomal homologous recombination, a plasmid containing duplicated copies of the gene coding for hygromycin resistance was introduced into the genome of a repair-proficient human cell line, KMST-6, and two repair-deficient lines, XP2OS(SV) from xeroderma pigmentosum complementation group A and XP2YO(SV) from complementation group F. Neither hygromycin-resistance gene codes for a functional enzyme because each contains an insertion/deletion mutation at a unique site, but recombination between the two defective genes can yield hygromycin-resistant cells. The rates of spontaneous recombination in normal and xeroderma pigmentosum cell strains containing the recombination substrate were found to be similar. The frequency of UV-induced recombination was determined for three of these cell strains. At low doses, the group A cell strain and the group F cell strain showed a significant increase in frequency of recombinants. The repair-proficient cell strain required 10- to 20-fold higher doses of UV to exhibit comparable increases in frequency of recombinants. These results suggest that unexcised DNA damage, rather than the excision repair process *per se*, stimulates such recombination.

Cells from persons with an inherited predisposition to skin carcinomas on sunlight-exposed areas, xeroderma pigmentosum (XP) patients (1), are markedly deficient in nucleotide excision of DNA damage induced by UV radiation, sunlight, and chemicals that produce DNA lesions similar to those induced by UV (2). XP cells are abnormally sensitive to the cytotoxic, mutagenic, and transforming effects of such agents (3-7). Since protooncogenes can be activated by point mutations (8), the increased sensitivity of XP cells to mutation induction is considered to contribute to the abnormally high frequency of tumors in these patients. But recent evidence points to homologous recombination in somatic cells as another mechanism in the multistep process of tumorigenesis (9-11). For example, Cavanee *et al.* (9) showed that recombination in cells from a retinoblastoma patient allowed a mutant allele of the *RBI* locus to become homozygous, permitting expression of the recessive phenotype in the tumor cells. If unexcised DNA lesions promote homologous recombination, then XP cells could be prone to increased homozygosis of oncogenes through recombination.

Wang *et al.* (12) showed that DNA-damaging agents, including UV, can induce a dose-dependent increase in the frequency of intrachromosomal homologous recombination in mouse cells in culture, and Bhattacharyya *et al.* (13) showed that the increase correlated with the number of DNA

adducts formed. However, there were significant differences between agents in the frequency of recombination induced per adduct, and these could not be explained by differences in overall rate of excision of the adducts formed. From this study it was not possible to determine whether the recombination resulted from unrepaired adducts or from the process of excision repair itself. To answer these questions, we have studied spontaneous and UV-induced intrachromosomal recombination in human cell lines that differ in their capacity for excision repair. We found that the strains have similar rates of spontaneous intrachromosomal recombination, but the frequency of UV-induced recombination in the XP cell strains is much higher than in the repair-proficient cell strain.

MATERIALS AND METHODS

Construction of pTPSN. A hybrid sequence consisting of the promoter of the Herpes simplex virus 1 thymidine kinase (*Htk*) gene, the coding region of the gene coding for hygromycin (*Hyg*) resistance (*hyg*), and the poly(A) site of the *Htk* gene was constructed by replacing a *Bgl* II-*Sma* I fragment of the *Htk* gene in pTK173 (nucleotides 460-1625; ref. 14) with a 1.3-kilobase-pair (kbp) *Hind*III-*Bgl* II fragment containing the coding region of the *hyg* gene (15). This hybrid *hyg* gene sequence was shown by transfection to be functional in mammalian cells. The first insertion/deletion mutation was introduced into the *hyg* gene at the unique *Pvu* I site (nucleotide 594; ref. 15) by resection with T4 DNA polymerase and ligation of a 10-bp *Hind*III linker (see Fig. 1). The second insertion/deletion mutation was introduced at a *Sac* II site (nucleotide 1011; ref. 15) by resection with T4 DNA polymerase and addition of a 10-bp *Hind*III linker. The first mutant gene as a 2.2-kbp *Pvu* II fragment was cloned into the unique *Sca* I site of plasmid pJS-1 (16). The second mutant, also on a 2.2-kbp fragment, was cloned into pJS-1 at the unique *Bam*HI site using *Bam*HI linkers. The two mutations are located 417 bp apart in the *hyg* gene. The two genes are distinguishable in Southern blot analysis because the *Pvu* I mutant *hyg* gene is recoverable on a 2.9-kbp *Dra* I fragment, whereas the *Sac* II mutant *hyg* gene is recoverable on a 2.6-kbp *Dra* I fragment or on a 2.2-kbp *Bam*HI fragment (Fig. 1).

Cells and Culture Conditions. The KMST-6 cell line (17), from M. Namba (Kawasaki Medical School, Kawasaki, Japan), and the simian virus 40-immortalized XP cell lines [XP2OS(SV), complementation group A, and XP2YO(SV),

Abbreviations: *Htk*, Herpes simplex virus 1 thymidine kinase gene; G418^r, G418 resistant; *Hyg*, hygromycin; *Hyg*^r, *Hyg* resistant; *hyg*, gene coding for *Hyg* resistance; *neo*, gene coding for G418 resistance; XP, xeroderma pigmentosum.

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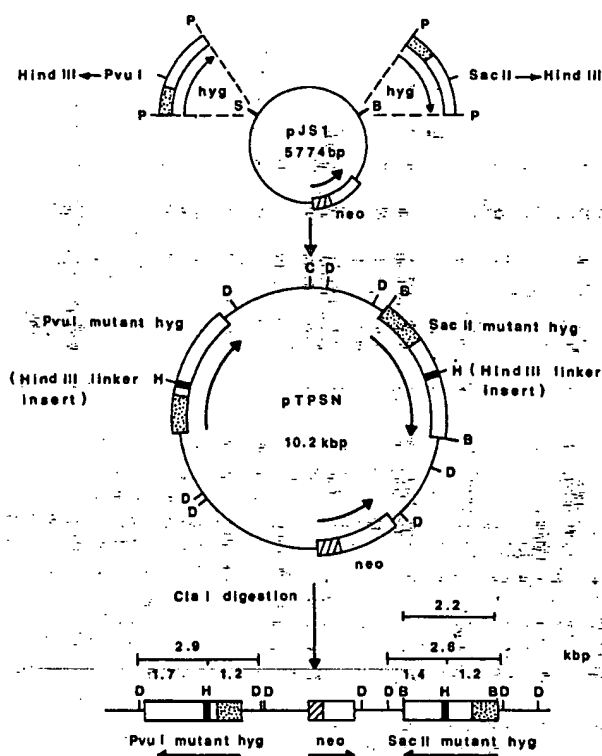


FIG. 1. Construction of pTPSN showing orientation of the two *hyg* genes and of the *neo* gene and the size of the restriction enzyme fragments. B, *Bam*HI; C, *Cla*I; D, *Dra*I; H, *Hind*III; P, *Pvu*II; S, *Sca*I. The stippled or hatched areas indicate gene promoter regions. The arrows indicate the direction of transcription.

group F (18)], supplied by H. Takebe (Kyoto University, Kyoto, Japan), were cultured in Eagle's minimal essential medium with 10% (vol/vol) fetal bovine serum and modified as in ref. 12.

DNA Transfection and Isolation of G418-Resistant (G418^r) Transfectants. Cells were transfected as described (19) and selected with 200 μ g of active G418 (GIBCO) per ml of medium 24 hr later. After 3 weeks, colonies were transferred to small wells and propagated in selective medium.

Southern Blot Analysis. DNA was isolated and digested with *Bam*HI, *Dra*I, and *Hind*III using the supplier's (New England Biolabs) recommended conditions, and Southern blotting analysis using the *hyg* gene as probe was carried out as described (19).

Optimizing Conditions for Selection with Hyg. A concentration of Hyg (Calbiochem) 2–3 times higher than that required to reduce the survival of the nontransfected parental cell lines to <0.01% of the untreated control was determined empirically to be 30 μ g/ml for KMST-6-9, 100 μ g/ml for XP2OS(SV)-18, and 80 μ g/ml for XP2YO(SV)-65. The effect of cell density on the recovery of Hyg-resistant (Hyg^r) colonies was determined by plating each cell strain at 0.3×10^6 to 2×10^6 cells per 100-mm diameter dish. Sixty Hyg^r cells taken from recombinant colonies derived from the corresponding cell strain were added into half of the dishes and selection with Hyg was begun 24 hr later and continued for 3 weeks, with fresh culture medium containing Hyg added every 5 days. Recovery was determined from the number of colonies formed, corrected for the spontaneous background. These reconstruction studies indicated that densities of 3×10^5 to 7×10^5 cells per dish were optimal. Electronic cell counting revealed that cell attachment ranged from 70% to 95%. For each subsequent experiment this number was

determined empirically at the time of carcinogen treatment to determine the number of target cells present.

Determination of Rate of Spontaneous Recombination. The rate was determined using fluctuation analysis (20). For each assay, 10–20 parallel cultures of 300 cells were plated into small wells and propagated to 5×10^6 or 1×10^7 cells. Approximately 1×10^6 cells from each of these subcultures were plated at 5×10^5 cells per 100-mm diameter dish. Selection with the designated concentration of Hyg was begun after 18 hr. Representative colonies were isolated for further characterization. The rest were stained and the rate of recombination was calculated (21).

Reconstruction Studies to Determine the Recovery of UV-Induced Recombinants. A lawn of each cell strain (5×10^5 cells per dish) was plated into 20 dishes. Ten dishes of each set were seeded with 60 Hyg^r cells from recombinant clones derived from the corresponding cell strain. The other 10 had only the lawn. One-half of each series was exposed to a dose of UV known to give 40–50% survival and, after 24 hr, the cells were selected with Hyg. After 3 weeks, the dishes were stained and the fraction of Hyg^r cells recovered as colonies was determined.

Assay for UV-Induced Cytotoxicity and Recombination. Exponentially growing cells were plated at 5×10^5 cells per dish. Sufficient dishes were used for each dose so as to have 1.5×10^6 surviving target cells. An extra set of cells plated at cloning densities was used to assay survival. The cells were irradiated as described (6) 15 hr later. Cells plated at low density were allowed 14 days to form macroscopic colonies, with one refeeding. The rest were selected with Hyg. After 3 weeks, clones were counted and representative recombinants were isolated for characterization. The frequency of recombination was determined from the number of Hyg^r colonies corrected for the number of viable cells. The frequency of induced recombination was determined by subtracting the background frequency observed in the control population.

Determination of Unscheduled DNA Synthesis. Cells were plated into two sets of 35-mm-diameter dishes (at 1×10^5 cells per dish) and, after 24 hr, the medium was exchanged for culture medium containing or lacking hydroxyurea (10 mM). Then 30 min later the cells were rinsed with isotonic phosphate-buffered saline and irradiated with 15 J/m² or not irradiated, refed with their respective medium supplemented with [³H]thymidine (10 μ Ci/ml, 80 Ci/mmol; 1 Ci = 37 GBq), and incubated at 37°C for 3 hr. The UV-induced incorporation of [³H]thymidine was determined (22) from the counts in the irradiated cultures compared to the control, taking into account the inhibitory effect of hydroxyurea on S-phase incorporation. As a second method, cells were seeded into dishes containing coverslips, irradiated with 15 J/m² after 24 hr, and allowed to incorporate [³H]thymidine for 3 hr. The coverslips were processed for autoradiography and analyzed automatically (23).

RESULTS

Construction and Characterization of the Human Cell Strains Containing the Recombination Substrate. The substrate used for previous studies on carcinogen-induced homologous recombination (12, 13) contains duplicated copies of the (*Hrk*) gene, and this requires that the host cells be thymidine kinase-deficient. To broaden the range of lines that could be employed in the study of the role of DNA repair in such recombination, we constructed a plasmid, pTPSN, containing duplicated copies of the dominantly acting *hyg* gene, each inactivated by an insertion/deletion mutation generating unique *Hind*III sites (Fig. 1). The nature of these mutations makes reversion highly unlikely. The *hyg* genes are in direct-repeat orientation and flank the dominantly acting gene coding for G418 resistance (*neo*). pTPSN was linearized

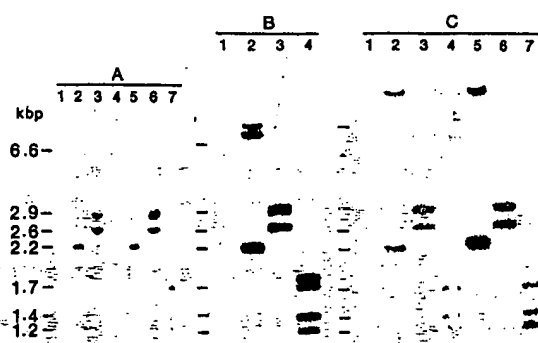


FIG. 2. Southern blot analysis of genomic DNA from various transfectants to determine the number and integrity of the recombination substrate. To determine the number of copies, the DNA was digested with *Bam*HI (lanes A2, A5, B2, C2, and C5). To determine if the two *hyg* genes were intact, the DNA was digested with *Dra*I (lanes A3, A6, B3, C3, and C6). To see if the linker insert was present, the DNA was digested with *Dra*I and *Hind*III (lanes A4, A7, B4, C4, and C7). As a control, DNA from nontransfected parental cells was digested with *Bam*HI (lanes A1, B1, and C1). Lanes: A1, KMST-6; A2-A4, KMST-6-9; A5-A7, KMST-6-19; B1, XP2OS(SV); B2-B4, XP2OS(SV)-18; C1, XP2YO(SV); C2-C4, XP2YO(SV)-51; C5-C7, XP2YO(SV)-65.

at its unique *Cla*I site to facilitate integration of the two *hyg* genes in the proper configuration and introduced into repair-proficient cell line KMST-6 and XP cell lines XP2OS(SV) and XP2YO(SV). The frequency of G418^r transfectants per 1×10^5 cells per μ g of DNA was 11 for KMST-6; 9.8 ± 6.9 for XP2OS(SV); and 8.1 ± 4.1 for XP2YO(SV).

Representative G418^r transfectant cell strains from each parental line were isolated, tested for the ability to yield Hyg^r recombinants, and analyzed by Southern blotting to determine the number of copies of the plasmid and to examine the integrity of the *hyg* genes. As shown in Fig. 1, digestion with *Bam*HI should produce a 2.2-kbp band and one or more larger junction fragments containing the other *hyg* gene. If only a single integrated copy of the substrate is present, one junction fragment band will be seen; if two copies of the substrate have integrated at separate sites, there will be two junction fragments, etc. If the *hyg* genes are intact, digestion with *Dra*I should produce two bands (2.6 kbp and 2.9 kbp). Digestion with *Dra*I and *Hind*III should produce a 1.7-kbp band, a 1.4-kbp band, and a 1.2-kbp band composed of two fragments. The *Bam*HI data suggested that KMST-6-9 and XP2YO(SV)-65 contain a single copy of the plasmid; KMST-6-1, XP2OS(SV)-18, and XP2YO(SV)-51 have two copies; and KMST-6-19 has three copies. Digestion with *Dra*I showed that the *hyg* genes were intact in all of these strains except XP2OS(SV)-18, which had an extra band, larger than

the two expected bands. Digestions with *Dra*I and *Hind*III indicated that all the *hyg* genes contained the *Hind*III linker insertion mutation. Examples of such analyses are shown in Fig. 2. The fraction of suitable G418^r transfectants from each of the human cell strains (i.e., having a low number of copies of the plasmid and productive for Hyg^r colonies) was similar.

Six of these suitable cell strains were further tested for their rate of spontaneous recombination by using fluctuation analysis tests. The results are indicated in Table 1. The rates of spontaneous recombination per 1×10^6 cells per generation ranged from 2.1 ± 1.0 to 10.5 ± 3.0 , with a mean value of 4.6 for the KMST-6-derived cell strains, a value of 8.3 for XP2OS(SV)-18, and a mean value of 6.5 for the XP2YO(SV)-derived cell strains.

Relative UV Sensitivity of the Cell Strains and Their Excision Repair Capacity. From each of the three parental cell lines, a transfectant cell strain with a low copy number of the substrate was chosen for study. Their sensitivity to UV was determined from loss of colony-forming ability. As shown in Fig. 3 Upper, the XP cell strains were significantly more sensitive to UV than the KMST-6-derived cells. The curve for the latter is comparable to that of diploid fibroblast cell lines derived from normal persons (5-7).

The relative capacity of these cell strains to carry out UV-induced unscheduled DNA synthesis was compared using two methods. The extent of UV-induced incorporation of [³H]thymidine (dpm per 1×10^5 cells) was 70×10^3 for KMST-6-9 cells, 7×10^3 for the XP2YO(SV)-65, and 11×10^3 for the XP2OS(SV)-18 cells. The mean numbers of silver grains observed per nucleus were consistent with these results (i.e., 35.2 ± 9.1 , 8.2 ± 4.8 , and 7.1 ± 4.9 , respectively).

Optimization of Conditions for Measuring the Frequency of UV-Induced Recombination. Cells from each cell strains were plated, irradiated with doses giving 40% survival, and selected with Hyg after various expression periods. The results (data not shown) indicated that selection could be started as early as 12 hr after treatment or as late as 48 hr. Therefore, a 24-hr expression period was used. Reconstruction studies to determine the recovery of recombinants under the experimental conditions involving UV indicated that, when the fraction of cells that attached (70%-95%) was taken into account, recovery was 100%. Reconstruction studies also showed no difference between Hyg-sensitive cells and Hyg^r cells in sensitivity to UV.

Frequency of Homologous Recombination Induced by UV Radiation. As shown in Fig. 3, the XP cell strains exhibited a dose-dependent increase in the frequency of Hyg^r recombinants at low doses (i.e., 0.1-1.0 J/m²); the excision repair-proficient KMST-6-9 cell strain required 10- to 20-fold higher doses of UV radiation to exhibit comparable increases in recombination. These results suggest that unexcised DNA damage induced by UV radiation, rather than the nucleotide

Table 1. Rate of spontaneous recombination in various cell strains

Cell donor	G418 ^r transfectants tested	Plasmid copy number	Recombination per 1×10^6 cells per cell generation	Hyg, μ g/ml
Normal	KMST-6-1	2	2.8	75
	KMST-6-9	1	$2.1 \pm 1.0^*$	50
	KMST-6-19	3	9.9	75
XP group A	XP2OS(SV)-18	2	8.3 ± 1.1	100
XP group F	XP2YO(SV)-51	2	10.5 ± 3.0	100
	XP2YO(SV)-65	1	$2.5 \pm 2.4^\dagger$	100

G418^r transfectant cell strains yielded Hyg^r clones and, therefore, were tested for rate. Recombination values have not been divided by the plasmid copy number.

*This value takes into account that recombinants of this particular cell strain exhibited 70% survival at a Hyg concentration of 50 μ g/ml.

†This value takes into account that recombinants of this cell strain exhibited 95% survival at a Hyg concentration of 100 μ g/ml.

excision repair process *per se*, stimulates homologous recombination.

Characterization of Recombination Products. The types of recombination products induced by UV in these cells were compared with the spontaneous recombination products. For KMST-6-9 and XP2YO(SV)-65 cells, which contain only a single copy of the plasmid, the ratio of gene conversions to single reciprocal exchanges can be determined by assaying the recombinants for G418 resistance. A single reciprocal exchange results in a single wild-type copy of the *hyg* gene and loss of the *neo* gene (Fig. 4 Upper). In contrast, a gene conversion event preserves the *hyg* gene duplication and the *neo* gene. Results of the biological assay with these two cell strains suggested that all of the events involved gene conversion. To confirm the bioassay in single-copy strains and to determine the ratio of gene conversion to reciprocal exchange in multicopy strains, Southern blot analysis was performed. The results (Table 2 and Fig. 4) showed that all but one of the events involved gene conversions. In general, except for strain XP2YO(SV)-65, there was a bias toward conversion of the *Pvu* I mutant gene in both the spontaneous and the UV-induced gene conversion.

DISCUSSION

We have investigated the role of UV-induced DNA damage and nucleotide excision repair in intrachromosomal homologous recombination, using a cell strain with a normal capacity for nucleotide excision repair and two repair-deficient XP cell strains representing complementation groups A and F.

Six cell strains with low copy numbers of intact substrate were tested by fluctuation analysis to determine rates of spontaneous recombination. The rates for the three XP strains did not differ significantly from the rates for the normal strains (Table 1), indicating that the repair deficiencies in these XP cell strains had no detectable effect on the

spontaneous rate of intrachromosomal recombination. In addition, these results support the hypothesis that spontaneous recombination does not result from the presence of DNA damage that resembles the lesions induced by UV. Furthermore, the types of recombination products (i.e., gene conversion versus reciprocal exchange) recovered from normal and XP cells were similar.

In concluding that the DNA repair deficiencies of the XP cell strains did not affect the types of recombination products obtained, it must be pointed out that in the present study, essentially all the *hyg* gene recombinants had patterns consistent with gene conversion (Table 2). In contrast, in studies of spontaneous (16, 20) or carcinogen-induced intrachromosomal homologous recombination (12, 13) using mouse L cells containing a plasmid with duplicated copies of the *Htk* gene, the ratio of gene conversion events to single reciprocal exchanges between the *Htk* genes was 85:15. This difference may reflect the difference in cell strains or in recombination substrates or an inability to recover the reciprocal recombination product. In reciprocal recombinants, the *hyg* gene lacks the *neo* gene enhancer, and it is possible that the level of *hyg* expression in such recombinants is inadequate. The possibility that lower levels of expression are responsible for the observed low frequency of reciprocal recombinants with *hyg* genes is strengthened by results using the *Htk* gene as a

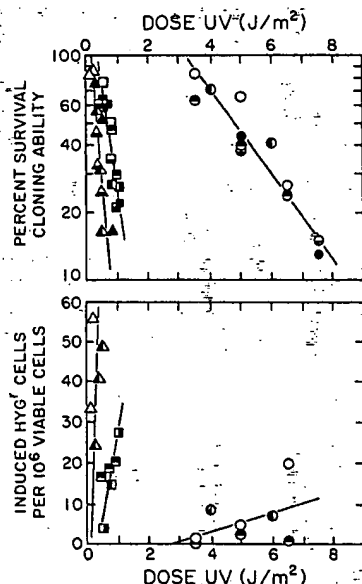


FIG. 3. Cell killing (Upper) and induction of recombination (Lower) as a function of UV dose. Triangles, XP2OS(SV)-18; squares, XP2YO(SV)-65; circles, KMST-6-9. (Upper) Data (i) from experiments in which only the cytotoxic effect of UV was measured as well as (ii) from the survival results from the recombination assays (Lower). Background frequencies have been subtracted. These values per 1×10^6 cells were as follows: 31 (Δ), 34 (Δ), 9.4 (\square), 28 (\circ), 43 (\square), 5.6 (\circ), 22 (\circ), 30.6 (\circ).

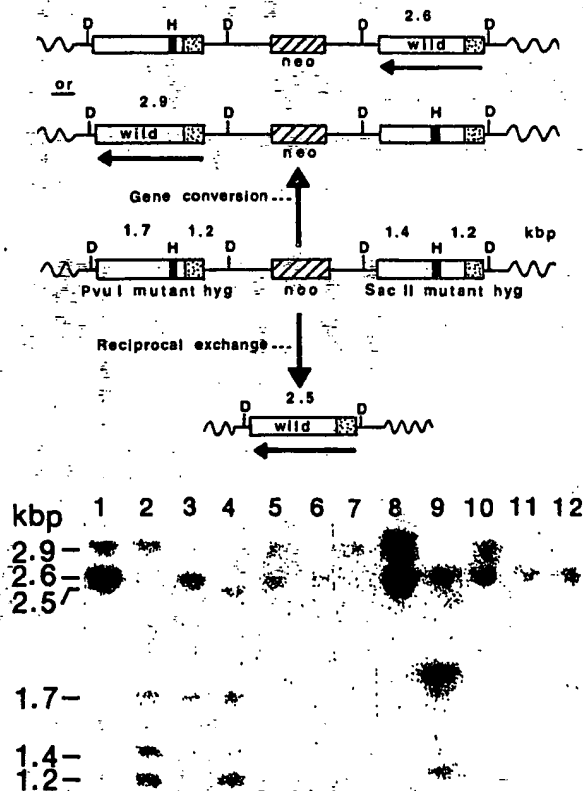


FIG. 4. Southern blot analysis of DNA from various *Hyg*^r recombinants and their corresponding *Hyg*-sensitive cell strains to determine the type of recombinational product. (Upper) Kinds of recombination products generated by gene conversion or reciprocal exchange. Abbreviations are as in Fig. 1. (Lower) Southern blot analysis. Lanes: 1-4, KMST-6-1 and three of its recombinants; 5-7, KMST-6-9 and two of its recombinants; 8 and 9, XP2OS(SV)-18 and one of its recombinants; 10-12, XP2YO(SV)-65 and two of its recombinants. The DNA in the first lane of each set was digested with *Dra* I, and that in each of the other lanes of a set was digested with *Dra* I and *Hind*III to determine which mutant *hyg* gene was converted to wild type (*Hind*III resistant).

Table 2. Molecular characterization of independent spontaneous and UV-induced Hyg^r recombinants from each cell strain

Cell strain	No. of recombinants tested	Gene conversion, no.		Single reciprocal exchange, no.
		<i>Pvu</i> I mutant	<i>Sac</i> II mutant	
Spontaneous*				
KMST-6-1	5	2	2	1
KMST-6-9	11	5	6	0
KMST-6-19	6	6	0	0
XP2OS(SV)-18	11	10	1	0
XP2YO(SV)-51	9	9	0	0
XP2YO(SV)-65	6	0	6	0
Total	48	32 (67%)	15 (31%)	1 (2%)
UV-induced†				
KMST-6-9	12	2	10	0
XP2OS(SV)-18	15	15	0	0
XP2YO(SV)-65	5	4	1	0
Total	32	21 (66%)	11 (34%)	0 (0%)

* Each spontaneous recombinant was isolated from an independent subpopulation derived from fluctuation tests. Each UV-induced recombinant was isolated from an independent population given the highest UV dose. Values in parentheses are percentage of total. A gene conversion in a *Pvu* I mutant is detected as a 2.9-kbp *Dra* I fragment; a gene conversion in a *Sac* II mutant is detected as a 2.6-kbp *Dra* I fragment.

substrate for intrachromosomal recombination in human cells showing that reciprocal-exchange products are found in 10% of the total spontaneous and UV-induced recombinants in both repair-proficient and -deficient strains (24).

XP cell strains exhibited a significant dose-dependent increase in the frequency of recombinants at low UV doses. With the normal cells, a 10- to 20-fold higher UV dose was required for a comparable increase in frequency. Of relevance, these normal cells exhibited a 10-fold higher rate of UV-induced unscheduled DNA synthesis (repair synthesis) than the XP cells. These data suggest that the excision repair functions that are defective in the XP cells are not required for UV-stimulated homologous recombination and support the conclusion that intrachromosomal recombination is stimulated by persistent UV-induced lesions. Such stimulation could be due to discontinuities in daughter-strand DNA near the site of the UV-induced lesions that could provide a substrate for initiating recombination. Our data for intrachromosomal recombination are consistent with extrachromosomal recombination studies showing that the frequency of recombination among UV-irradiated infecting virus is higher in excision repair-deficient human cells than in repair-proficient cells (25). DNA damage can enhance DNA-mediated transformation of human cells (26–29), but not rodent cell lines (29), probably by increasing the chance of integration of the transfected DNA. Therefore, it appears that persistent DNA damage can influence both homologous and nonhomologous recombination in human cells.

Finally, since mitotic recombination between homologous chromosomes can lead to homozygosity of recessive oncogenes, the results of our study suggest that the increased risk of certain malignancies in individuals with XP could reflect increased recombination after DNA damage.

We thank Dr. H. Takebe for the XP cell lines, Dr. M. Namba for the KMST-6 cell line, and Dr. J. Smiley (Department of Pathology, McMaster University, Hamilton, Ontario, Canada) for pTK173. We thank our colleague Dr. N. P. Bhattacharyya for helpful advice during the work and Mr. N. Davis for excellent technical assistance. This research was supported in part by Grants CA21253 CA48066, PO1-CA39238, and GM32741 from the National Institutes of Health. A.R.G. was supported by a National Science Foundation Graduate Fellowship.

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EXHIBIT 2

Somatic Cell and Molecular Genetics, Vol. 17, No. 3, 1991, pp. 463-469

Kinds and Locations of Mutations Arising Spontaneously in the Coding Region of the *HPRT* Gene of Finite-Life-Span Diploid Human Fibroblasts

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Received 22 April 1991—Final 11 June 1991

Abstract—Spontaneous thioguanine-resistant mutants were derived from populations of finite-life-span, diploid human fibroblasts by means of a fluctuation analysis. cDNA was prepared from mutant *HPRT* mRNA and amplified by the polymerase chain reaction, and the sequence of the product was analyzed. Exon deletions, which very likely arose from mutations in the intron splice site consensus sequences, were found in 10 of the 37 mutants examined (27% of the total). Among the 28 mutations in the coding sequence, base pair substitutions predominated (89%). With the exception of one base pair involved in a tandem mutation, all base pair substitutions resulted in alterations in the predicted amino acid sequence of the protein. In addition there were three frameshift mutations, consisting of the deletion of one or two base pairs. Although mutations occurred throughout the coding sequence, 50% (14/28) were found in the 5' portion of exon 3.

INTRODUCTION

Populations of diploid human fibroblasts in culture exhibit a mutation rate at the X-linked hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) locus of approximately $6 \text{ per } 10^6 \text{ cells per cell generation}$ (1). These mutations, which are designated spontaneous mutations because they occur in the absence of any-known mutagenic agent, arise as a result of endogenous damage to DNA, e.g., deamination of cytosine, or errors introduced into the genome during the process of DNA synthesis. Attention recently has been focused on spontaneous mutations generated during cell replication because these may be implicated in the pathogenesis of human cancer (2-4). Yet knowledge of the nature of such spontaneous mutations in

endogenous genes of mammalian cells or of the molecular mechanisms that result in such mutations is still limited.

Molecular genetic analysis of spontaneous mutagenesis in such cells began with the study of exogenous genes that have been integrated into the genome of rodent cell lines (5-7). Gross alterations, i.e., deletions and insertions, were the most abundant kind of mutations observed in those assays. DNA sequence analysis of spontaneous mutations arising in an endogenous mammalian gene has been reported from the laboratory of Glickman (8) and Meuth (9, 10). These investigators examined the endogenous adenine phosphoribosyltransferase (*aprt*) locus of an infinite-life-span rodent cell line (CHO), utilizing restriction digest subcloning strategies or polymerase chain reaction (PCR)

amplification of genomic DNA to obtain mutant DNA for sequence analysis. Their studies showed that the mutations were primarily base pair substitutions. However, the mutational spectra found in the two laboratories did not agree with each other. In particular, one position at which 23% of the base pair substitutions were located in a study of 30 mutants (8) was not found to be altered in two other studies, which examined a total of 120 mutants (9, 10).

Before the advent of the PCR technique, which allows exponential amplification of a specific sequence with known flanking regions, it was not feasible to determine the nature of the mutations arising spontaneously during extended cell replication in finite-life-span cells. This is because the number of population doublings required for a fluctuation analysis test (11), followed by expression of G-thioguanine (TG) resistance and the formation of a macroscopic clone, brought the cells of interest to near the end of their in vitro life-span. The cells usually could not replicate a sufficient number of additional generations to provide the large number of cells required for genomic DNA or RNA extraction. However, Yang et al. (12) described a method using PCR to amplify cDNA complementary to mutant *HPRT* mRNA directly from the lysate of a very small number of such cells (100–200). This method has been used to analyze the nature of mutations induced by various agents in the coding region of the endogenous *HPRT* locus of diploid human fibroblasts (13–16). In the current study we report the results of DNA sequence analysis of the coding region of the *HPRT* gene of 37 independent TG-resistant mutants that arose in populations of fibroblasts that had been expanded 2^{15} -fold in a fluctuation analysis test (11). Ten mutants were missing an exon; three had a deletion of one or two base pairs; 23 had a single base pair substitution; and one had a tandem substitution. The base substitutions were all

types except A \rightarrow T \rightarrow T \rightarrow A. No single predominant mechanism could explain the types of changes seen. The results provide a data base for comparison with induced mutational spectra at this locus.

MATERIALS AND METHODS

Cells and Culture Conditions. A finite-life-span diploid human fibroblast cell line explanted from neonatal foreskin (17), designated SL89, was used for generating and selecting TG-resistant cells. Early passage cells were used and were routinely cultured in a modified MCDB-110 medium (18) containing 10% supplemented bovine calf serum (Hyclone, Logan, Utah) (culture medium). For selection of TG-resistant cells, the same medium, but lacking adenine and containing 5% fetal bovine serum, 5% supplemented calf serum, and 40 μ M TG was used (selection medium). Cell culture rooms and hoods were illuminated with yellow lights to avoid induction of DNA lesions.

Generation and Selection of TG-Resistant Cells. Each spontaneous TG-resistant clone was obtained by establishing a series of independent cultures of 10^3 cells each and allowing these cells to replicate through 15 population doublings to yield $\sim 3 \times 10^7$ cells. In selection medium 1×10^6 cells from each culture were reseeded at 500 cells/cm², and the cloning efficiency of the cells was also determined by plating at low density. When macroscopic TG-resistant colonies had developed 14 days later, these were located and isolated. Since the cells in each clone had undergone about 40 population doublings since the beginning of the fluctuation test assay, their ability to be expanded further was limited. Therefore, *HPRT* cDNA was amplified directly from lysates of the cells in each individual clone.

Synthesis of First-Strand cDNA Directly from mRNA in Cells. Clones, consisting of 100–500 cells, were trypsinized and sus-

pended in 0.5 ml ice-cold, RNase-free phosphate-buffered saline, pH 7.4, and centrifuged in an RNase-free 0.5-ml Eppendorf tube for 10 min at 4°C. The supernatant was removed, and the cell pellet was resuspended in 5 µl of the cDNA cocktail described by Yang et al. (12). The reverse transcriptase reaction was performed at 37°C for 1 h to allow the cell membranes to be lysed by detergent and first-strand cDNA to be synthesized from total cytoplasmic poly(A) mRNA.

Amplification of *HPRT* cDNA and DNA Sequencing. The experimental conditions, optimized for preparing second-strand *HPRT* cDNA, amplifying the cDNA 10¹¹-fold using two sets of primers and two PCR stages of 30 cycles each, and sequencing the products directly using three sequencing primers and a modified Sanger dideoxynucleotide procedure have been described (12).

RESULTS AND DISCUSSION

Mutant Frequency. A population of diploid human cells, with a background frequency of TG-resistant cells of less than 1×10^{-5} was distributed into six independent cultures composed of 10³ cells/35-mm dish, and the cells were propagated through 15 population doublings to approximately 3×10^7 cells/culture. The probability that an initial culture of 10³ cells contained a preexisting background mutant was less than 1/100. At the end of the expansion, 10⁶ cells from each culture were assayed for TG resistance. The mutant frequency observed was $5.5 \pm 5/10^6$ clonable cells (range 1.1–14.0). The cloning efficiency ranged from 42% to 60%.

Nucleotide Sequence Alterations. Table 1 lists the kinds of mutations observed and their locations in the coding sequence of the human *HPRT* gene. Among the 37 mutants sequenced, 10 (27%) exhibited a missing exon. Although there are no published studies of the DNA sequence of spontaneous

HPRT mutants in human cells for direct comparison, the proportion of missing exons among spontaneous *aprt* mutants in the rodent CHO cell line has been reported to range from 10% (15/150) (19) to 100% (3/3) (20). A recent analysis of spontaneous *APRT* mutants in a human colon cancer cell line (SW620) indicated that 20% (6/30) had splice site alterations (21). In addition, among *HPRT* mutants induced by UV light in populations of human fibroblasts, 26% had missing exons (16). The relatively high proportion of missing exons found in these studies may reflect, to some extent, the stringency of selection with purine analogs, but splicing errors are likely to be important in spontaneous and induced mutagenesis. The molecular nature of the mutations responsible for these missing exons was not evaluated in the present study since we have analyzed the sequence of the coding region, but such missing exons are likely to have resulted from point mutations in the splice site-consensus sequences, rather than from large deletions within the gene. This assumption is supported by the fact that among 150 *aprt* mutants arising spontaneously in populations of CHO cells, all of the mutations found were either in the coding region or in the 5' splice sites when the genomic DNA of that locus was sequenced (19).

Among the 27 mutants in our study that exhibited a mutation in the coding sequence, three contained a deletion of one or two base pairs, and the rest contained base substitutions, mainly single base substitutions. At least two of these deletions might have resulted from strand slippage during DNA replication, since the surrounding sequences contained two base pair repeats (22). Such structures were also found to flank deleted sequences in spontaneous *aprt* mutants that arose in rodent cells (19).

The 24 mutants analyzed that exhibited base pair substitutions included a tandem alteration, for a total of 25 substitutions. All but one base substitution, in a mutant that

Table 1. Kinds and Locations of Mutations Arising Spontaneously in Coding Region of *HPRT* Gene of Finite-Life-Span Human Fibroblasts

Mutant	Position	Exon	Type of mutation	5' → 3' Surrounding sequence	Amino acid change
I. Base substitutions					
SP27	106	2	T-A → C-G	GTG TTT ATT	Phe → Leu
SP4	142	3	C-G → A-T	GAA CGT CTT	Arg → Ser
SP9	178	3	C-G → T-A	GGC CAT CAC	His → Tyr
SP35	178	3	C-G → T-A	GGC CAT CAC	His → Tyr
SP30	186	3	T-A → G-C	CAC ATT GTA	Ile → Met
SP7	193	3	C-G → T-A	GCC CTC TGT	Leu → Phe
SP20	193	3	C-G → T-A	GCC CTC TGT	Leu → Phe
SP21	193	3	C-G → T-A	GCC CTC TGT	Leu → Phe
SP22	197	3	G-C → C-G	CTC TGT GTG	Cys → Ser
SP26	197	3	G-C → C-G	CTC TGT GTG	Cys → Ser
SP6	202	3	C-G → T-A	GTG CTC AAG	Leu → Phe
SP29	220	3	T-A → C-G	AAA TTC TTT	Phe → Leu
SP1	221	3	T-A → C-G	AAA TTC TTT	Phe → Ser
SP16	226	3	G-C → A-T	TTT GCT GAC	Ala → Thr
SP19	231	3	C-G → G-C	GCT GAC CTG	Asp → Glu
SP36	242	3	A-T → C-G	GAT TAC ATC	Tyr → Ser
SP12	364	4	C-G → T-A	GAT CTC TCA	Leu → Phe
SP18	470	6	T-A → C-G	AAG ATG GTC	Met → Thr
SP8	471	6	G-C → A-T	AAG ATG GTC	Met → Ile
SP11	471	6	G-C → T-A	AAG ATG GTC	Met → Ile
SP24	473	6	T-A → C-G	ATG GTC AAG	Val → Ala
SP10	494	7	T-A → G-C	CTG GTG AAA	Val → Gly
SP33	498	7	A-T → G-C	AAA AGG ACC	No Change
	499		A-T → G-C	AAA AGG ACC	Arg → Gly
SP5	586	8	A-T → G-C	TAT AAT GAA	Asn → Asp
II. Deletions					
SP15	272	3	G Deletion	GAT AGA TCC	Frameshift
SP17	438	6	G-C } Deletion	TTG CTT TCC	Frameshift
	439	6	C-G }		
SP23	564	8	T Deletion	TTT GTT GTA	Frameshift
III. Putative splice site mutations					
Mutant	Missing exon	Mutant	Missing exon	Mutant	Missing exon
SP3	2	SP-32	5	SP-13	8
SP28	4	SP-34	5	SP-14	8
SP31	4	SP-37	7	SP-25	8
SP2	4				

had two base substitutions in tandem, resulted in an alteration in the predicted amino acid sequence of the protein. As listed in Table 2 all types of substitutions except A · T → T · A transversions were observed, but transitions predominated, accounting for 68% of the substitutions. Transitions were also the most abundant type of spontaneous mutation in the rodent cell *aprt* gene, accounting for 61% (19) to 79% (8) of the total number of substitutions. Among transitions and transversions observed in the

present study, the mutations were more likely to involve G · C than A · T base pairs (ratio 56:44). In the *aprt* locus of rodent cell lines, bias toward spontaneous mutations involving G · C base pairs was even greater, i.e., 75% (10) to 93% (8) of the total number of base substitutions.

Several mechanisms have been suggested to account for the observed base substitutions, including deamination of 5'-methylcytosine or cytosine, oxidative damage, depurination and depyrimidination, and

Table 2. Types of Base Substitutions Arising Spontaneously in Coding Region of *HPRT* Gene of Diploid Human Fibroblasts

Type of base substitution	Number of base substitutions ^a
Transitions	
G·C → A·T	9 (36)
A·T → G·C	8 (32)
Transversions	
G·C → T·A	2 (8)
G·C → C·G	3 (12)
A·T → C·G	3 (12)
A·T → T·A	0 (0)
Total	25 (100)

^aNumbers in parentheses represent the percentage of the total number of base substitutions in each class of mutations.

^bincludes one tandem mutation.

errors introduced by DNA polymerases during DNA replication. It is unlikely that deamination of 5'-methylcytosine is involved in spontaneous mutagenesis at the human *HPRT* locus, since the mutations we observed did not occur at CpG methylation sites, and furthermore the active *HPRT* gene is only slightly methylated (23). Deamination of cytosine to yield uracil would result in G·C → A·T transitions if subsequent rounds of DNA replication occurred before the uracil was removed by mismatch repair processes or uracil glycosylase. de Jong et al. (8) suggested that the relatively low level of the latter enzyme in the CHO cell line they studied accounts for the high proportion (22/28, 79%) of G·C → A·T transitions found in the spontaneous base substitutions they analyzed. However, Phear et al. (10), using the same CHO cell line, found a much lower percentage of such transitions (22/55, 40%) among base substitutions in the spontaneous mutants in their study. Therefore, factors other than a low level of uracil glycosylase must be invoked to explain the high proportion of G·C → A·T transitions found by de Jong et al. (8). In the present study of human cells, which presumably have normal levels of uracil glycosylase, we also

found 36% of the base substitutions to be G·C → A·T.

Oxidative damage to DNA is another possible mechanism of spontaneous mutagenesis. Substances that produce superoxide-free radicals have been shown to be mutagenic (24, 25), and such free radicals are produced by a variety of cellular metabolic processes. One of the primary products of oxygen-free radical reaction with DNA is 8-oxoguanine (26), and in vitro studies showed that mammalian DNA polymerases selectively insert adenine across from such lesions (27), resulting in a G·A mispair. Targeted G·C → T·A transversions, which would result from such mispairing, have been found to be the predominant base substitution in 8-oxoguanine-substituted plasmids that replicated in *E. coli* (28). In contrast, when single-stranded M13 DNA was exposed to an oxygen-free radical generating system and allowed to replicate in SOS-induced *E. coli*, a variety of base substitutions were found (29). These primarily consisted of transversions involving guanine residues (56%, 50/87), but a significant number of G·C → A·T transitions were also found (23%, 20/87). It is difficult to assess the degree to which oxidative damage contributed to the kinds of mutations found in the present study, but the small number of transversions would suggest a limited role.

Depurination has been estimated to occur frequently, on the order of 2×10^4 bases per cell per day, and depyrimidination approximately 10 times less frequently (30). Although highly efficient repair processes exist to recognize, incise, and repair apurinic-apyrimidinic sites (AP) sites, it is estimated that approximately one site per cell per day escapes repair and represents a potentially mutagenic lesion (31). Studies of the replication of plasmid DNA by *E. coli* suggest that preferential insertion of dAMP across from an AP site occurs (32, 33). This would result in transversion mutations (G·C → T·A and A·T → T·A) in the case of depurina-

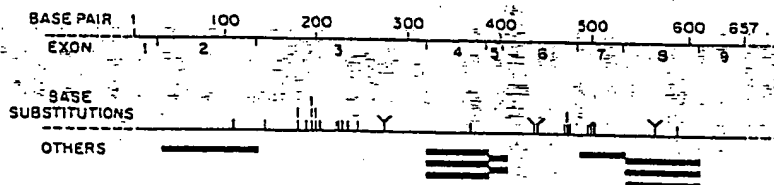


Fig. 1. Location of 37 independent *HPRT* mutations, which arose spontaneously in populations of diploid human fibroblasts, that had been subjected to a fluctuation test. Symbols: —, missing exons; |, base pair substitutions; Y, tandem mutation; and Y, base pair deletions.

tion, and a transition mutation ($G \cdot C \rightarrow A \cdot T$) in the case of depyrimidination. Transversions contributed to a minority of the mutations in the present study, suggesting a minor role for depurination. $G \cdot C \rightarrow A \cdot T$ transitions were the most common type of base substitution found, raising the possibility that depyrimidination played a role.

The fidelity of DNA polymerases may also contribute to the process of spontaneous mutagenesis. Although the error rates measured in vitro are far greater than those in vivo, results from a number of laboratories (summarized in 34) show that the most frequent errors are single base substitutions and that transitions are more common than transversions. This suggests that the mutations found in our present study primarily reflect errors introduced by the DNA polymerases during replication of the *HPRT* gene.

Several groups have studied spontaneous mutations arising in exogenous DNA integrated into the chromosomes of mammalian cells. In each case the predominant mutations were deletions of varying sizes (5-7). Since the exogenous sequences were intact when stably integrated, the predominance of deletion mutations, including elimination of the entire exogenous DNA sequence, indicates that cells handle such sequences differently from endogenous genes.

Although we found base pair alterations to be distributed throughout exons 2-8 of the *HPRT* gene, they were especially likely to occur in a 64-bp region located in the 5'

portion of exon 3 (Figure 1). This region, from position 178 to 242, contained 50% (14/28) of the mutations detected in the coding region, but comprised only 10% of the coding sequence. It is possible that alterations in this region of the human *HPRT* gene are particularly likely to affect protein function and, therefore, would be overrepresented among cells selected for resistance to TG. However, a highly conserved sequence in exons 4-5, composed of 60 bp, which contains a putative catalytic domain (35), had only one mutation in the present study. Furthermore, mutations induced by UV light give an entirely different spectrum (16), i.e., are not concentrated in the 5' half of exon 3.

In summary, we have presented data on the kinds and locations of mutations that arose spontaneously in the coding region of the human *HPRT* gene. Although no single mechanism emerges to explain the data, the analysis of the spectrum of spontaneous mutations will be useful for comparison with mutational spectra induced by chemical and physical agents.

ACKNOWLEDGMENTS

The expert technical assistance of Ena Zaccagnini and Andy Mendians is gratefully acknowledged. We thank Connie Williams for typing the manuscript. This research was supported in part by DHHS grant CA21253 from the National Cancer Institute. W.G.M. is the 1990 recipient of the Chevron-SRA Postdoctoral Fellowship in Risk Analysis.

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LETTERS TO NATURE

even on termini capped by a stretch of non-telomeric DNA, involves recombination, especially if the last few bases of the substrate are able to pair with C_1 - A sequence. Alternatively, if telomerase⁹⁻¹² exists in *Saccharomyces* and if it can use a substrate in which the telomeric sequences are subterminal, it could mediate those telomere formation events that do not involve recombination.

Our results indicate that telomere formation in yeast is accompanied by telomere-telomere recombination. This recombination requires surprisingly little homology, perhaps because it is a site-specific event promoted by the ability of telomere DNA to assume non-B form structures involving triplex or quadruplex associations^{13,14} or G-G¹⁵ or C-C base pairing¹⁶. The junction between telomeric and unique sequence DNA, an apparent hot spot of recombination *in vivo*, also acts as a boundary for Klenow DNA polymerase and S1 nuclease *in vitro*^{17,18}. Although the data here and elsewhere⁴ do not rule out alternative pathways for telomere formation in yeast, telomere-telomere recombination provides an efficient mechanism for immediate rescue of DNA termini with very short stretches of telomere DNA. In contrast, other events that alter telomere length in yeast do so gradually such that many generations are needed to see a change

in telomere length (reviewed in ref. 19). A working model for telomere formation in yeast by recombination is shown in Fig. 8. This scheme is reminiscent of the bacteriophage T4 'copy choice' model^{8,20} that allows complete replication of the T4 genome by a process that requires recombination. As this recombination is non-reciprocal and can result in a net increase in telomeric sequences it could contribute to telomere replication during the normal cell cycle.

If telomere-telomere recombination is important for telomere replication or maintenance, we also expect authentic telomeres to recombine. However, we did not detect transfer of C_4A_4 or C_4A_2 DNA to yeast chromosomes. These results can be explained if yeast telomeres are more likely to recombine with each other than with plasmid termini. This is possible as (1) chromosomal telomeres and internal stretches of C_1 - A are more abundant than plasmid termini, and (2) chromosomal telomeres have much greater homology to each other than to C_4A_4 or C_4A_2 DNA. Alternatively, if telomere-telomere recombination is a salvage pathway that acts only on those telomeres with very short stretches of C_1 - A DNA, its occurrence at natural chromosomes may be too rare to detect by Southern hybridization. □

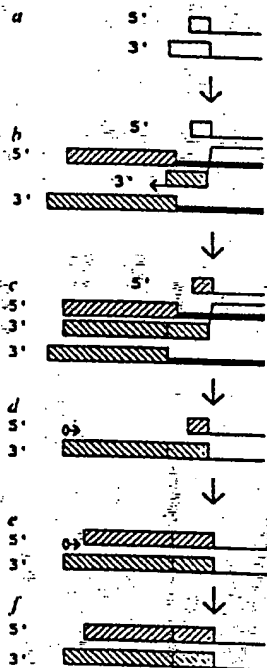


FIG. 3 Model for telomere formation by recombination. Different telomeric repeat sequences (thin-line hatched box) can promote telomere formation in yeast by serving as substrates for the addition of yeast C_1 - A repeats (thick-line hatched box). In both cases, the C-rich strand is marked with 5' and the G-rich strand is marked with 3'. Only one terminus of either a linear plasmid or a chromosome is shown: a. After replication of the plasmid in yeast and removal of the RNA primer, a single-strand tail is left at the 3' end of the newly replicated strand (represented by the unpaired thin-line hatched box). b. The 3' OH end of the single-strand G-rich tail invades another telomere (donor, represented by thick-line hatched box). At the donor end, most (or all) recombination events are initiated (or resolved) at the junction between telomeric DNA (thick-lined hatched box) and unique DNA (filled-in bar). c. The invading G-rich strand is extended by replication using the donor telomere as a template. d. After dissociation, the terminus carries an ~300 nucleotide long G₁₋₃T single-strand tail and serves as a template for primase (P) and conventional DNA polymerase mediated replication of the complementary strand (e). f. Subsequent removal of the RNA primer would still leave a gap at the 5' end of the newly replicated strand, but no sequence information would be lost. (Alternatively, the extended G-rich strand could fold back on itself to form a terminal hairpin and provide the primer for replication of the C-rich strand¹⁵.)

Received 21 December 1989; accepted 18 March 1990

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ACKNOWLEDGEMENTS. We thank G. Smith, A. Taylor, A. MacAuley and the members of our laboratory for comments on the manuscript. This work is supported by the NIH (V.A.Z.) and the Joan Taylor Fund (S.W.).

Telomeres shorten during ageing of human fibroblasts

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THE terminus of a DNA helix has been called its Achilles' heel¹. Thus to prevent possible incomplete replication² and instability^{3,4} of the termini of linear DNA, eukaryotic chromosomes end in characteristic repetitive DNA sequences within specialized structures called telomeres⁵. In immortal cells, loss of telomeric DNA due to degradation or incomplete replication is apparently balanced by telomere elongation⁶⁻¹⁰, which may involve *de novo* synthesis of additional repeats by a novel DNA polymerase called telomerase¹¹⁻¹⁴. Such a polymerase has been recently detected in HeLa cells¹⁵. It has been proposed that the finite doubling capacity

TABLE 1 Effect of donor age on telomere length in human fibroblasts

Cell strain	Age		Mean telomere length, kb \pm s.d. (n)
	In vivo years	In vitro MPD (MPD max)	
HSC172	Fetal	18-28 (88)	8.6 \pm 0.5 (3)
A30S	0	33 (58)	7.3 (1)
A38	24	31-33 (68)	6.9 \pm 0.3 (2)
A35	70	19 (41)	6.7 (1)
FOO1	71	21-29 (40)	6.5 \pm 0.4 (5)
FOO2	91	18-20 (45)	6.2 \pm 0.1 (3)

Mean telomere length (the length of the terminal restriction fragment) was determined as described in Fig. 2a for fibroblast cell strains at the earliest available mean population doubling (MPD) in separate experiments. Strains were derived from female fetal lung (HSC172), female newborn skin (A30S), male forearm skin (A38, A35) or female abdominal skin (FOO1, FOO2). MPD at time of assay and senescence (MPD max) are indicated. The correlation between increasing donor age and decreasing telomere length is statistically significant ($P < 0.05$).

of normal mammalian cells is due to a loss of telomeric DNA and eventual deletion of essential sequences^{1,16,17}. In yeast, the *est1* mutation causes gradual loss of telomeric DNA and eventual cell death mimicking senescence in higher eukaryotic cells¹⁷. Here, we show that the amount and length of telomeric DNA in human fibroblasts does in fact decrease as a function of serial passage during ageing *in vitro* and possibly *in vivo*. It is not known whether this loss of DNA has a causal role in senescence.

The finite replicative capacity of human fibroblasts in culture¹⁸ is well established and has been extensively used as a model of cellular senescence¹⁹. To investigate the effect of cell division and age on telomeric DNA, we grew nontransformed human fibroblasts *in vitro* until terminal passage and regularly determined the size distribution of terminal restriction fragments (that is, telomeres) by Southern blot analysis (Fig. 1). Mean telomere length decreased about 2 kilobases (kb) with cumulative population doublings in five strains of fibroblasts studied

(Fig. 2a). Moreover, the total amount of telomeric DNA also decreased (Fig. 2b), which indicates true loss of DNA and not simply rearrangement of telomeres with respect to restriction enzyme cleavage sites. Loss of telomeric DNA did not result from general degradation or loss of repetitive DNA in preparations of DNA from old cells²⁰, as other repetitive, non-telomeric sequence elements analysed in these experiments were not altered in size or amount (Fig. 1b, d). The loss was also not a function of growth state: cells at similar passage levels collected during exponential growth or during quiescence showed no significant difference in telomere distribution (not shown).

As loss of telomeric DNA observed with ageing *in vitro* may occur generally as somatic cells divide *in vivo*, it is important to determine the effect of donor age and tissue type on telomere length. Telomere length in fibroblasts varies between individuals and may become shorter during ageing *in vivo* (Figs 1 and 2, and Table 1). The increased heterogeneity of telomere lengths in cells from old donors (Fig. 1c, g) presumably reflects their variable history *in vivo* as well as their increased age in cell doublings. A larger population of individuals and other tissues must, however, be studied with both repetitive and unique-sequence telomere probes to confirm these observations. In addition, comparative studies should attempt to correlate telomere loss with cellular life span in different species.

The degree of telomere shortening observed during ageing of cultured human fibroblasts could be biologically significant. The ends of human telomeres consist of repeats of the sequence TTAGGG which are probably functionally important²¹. Human sperm telomeres have about 9 kb of TTAGGG repeats, whereas somatic cell telomeres may have only 4 kb. In addition, there seem to be variant forms of TTAGGG (such as TTGGGG) present sub-terminally²¹. The rates of loss of length and amount of telomeric DNA we have observed (Fig. 2) are in rough agreement with the estimate of 4 kb of TTAGGG per 'young' somatic telomere. Thus, the loss of 2 kb of terminal sequences could represent loss of most of the functional telomeric sequences. Even if there is substantially more than 4 kb of functional sequence at telomeres from young cells, this shortening

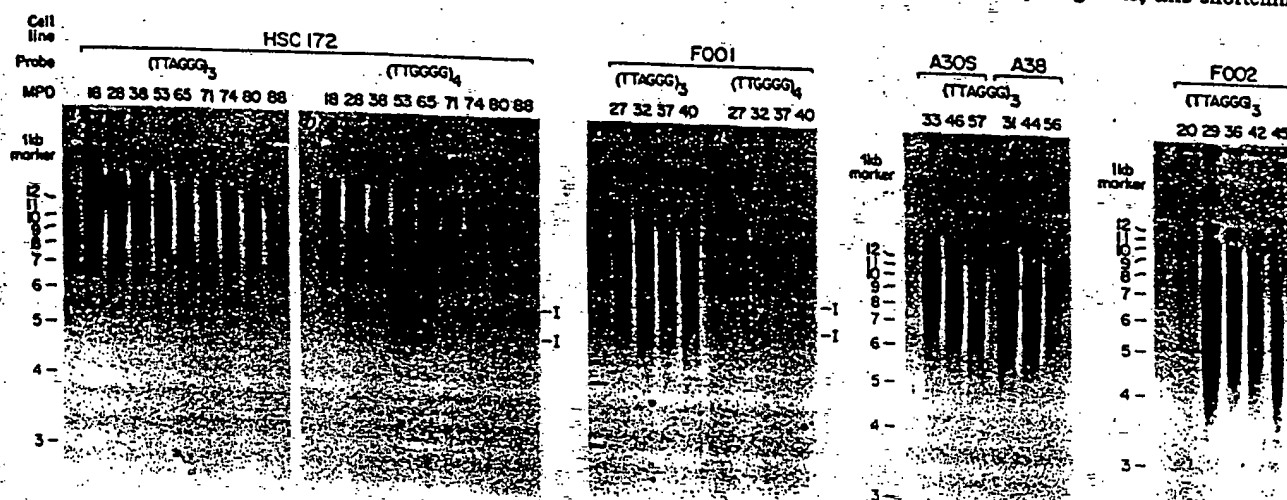


FIG. 1 Terminal restriction fragment (telomere) length in cultured human fibroblasts. Genomic DNA from fetal cell strain HSC172 (a, b), old adult FOO1 (c, d), FOO2 (g), newborn A30S (e) and young adult A38 (f) cell strains (see Table 1) was analysed at indicated mean population doublings (MPD) on Southern blots. Oligonucleotide probe (TTAGGG)₃ was used with high stringency washes to specifically detect telomeric sequences (a, c, e, f, g) and oligonucleotide (TTGGGG)₄ with low stringency washes to detect both telomeric sequences and internal repetitive sequences (b, d). The size (kb) and position of markers are indicated.

METHODS. Cells were grown and population doublings counted as described²¹ and DNA isolated when cells reached confluence. DNA was

digested to completion with restriction enzymes *MspI* and *RsaI* and carefully quantified by a fluorometric method²². A portion (1–2 μ g) was loaded onto a 0.7% agarose gel and resolved by electrophoresis at 1 V cm⁻¹ for 36–72 h. For FOO2, only 1 μ g was loaded in the first lane (MPD 20), 4 μ g in the second lane (MPD 29) and 2 μ g in each of the other lanes. DNA was depurinated by soaking gels in 0.1 M sodium citrate (pH 3.0) for 2 h, transferred to Nytran (Schleicher and Schuell) and hybridized in 6 \times SSC at 37 $^{\circ}$ C or 50 $^{\circ}$ C with end-labelled (TTAGGG)₃ or (TTGGGG)₄, respectively. Filters were washed in 3 \times SSC at 42 $^{\circ}$ C for the (TTAGGG)₃ probe or 4 \times SSC at 50 $^{\circ}$ C for the (TTGGGG)₄ probe.

could still be significant. As each cell contains 92 telomeres and the distribution of telomere lengths is wide, a loss of 2 kb from the mean length may imply a large increase in the proportion of cells missing TTAGGG from at least one telomere. The loss of even one telomere could cause the permanent cell-cycle arrest and chromosomal instability characteristic of senescent fibroblasts²³. Most significantly, chromosomal abnormalities increase dramatically in the terminal phase of fibroblast cultures²³ and about 90% of these are dicentric attached at their telomeres²⁴. Loss of critical telomere sequences in late-passage fibroblasts could explain these data. Whether loss of telomeric DNA *in vivo* might contribute to the elevated (but still relatively low)

frequency of chromosomal rearrangements, particularly dicentrics, observed in older humans^{25,26} is uncertain.

Tumour cells are also characterized by shortened telomeres^{27,28} and increased frequency of aneuploidy, including telomeric associations²⁸. If loss of telomeric DNA ultimately causes cell-cycle arrest in normal cells, the final steps in this process may be blocked in immortalized cells. Whereas normal cells with relatively long telomeres and a senescent phenotype may contain little or no telomerase activity, tumour cells with short telomeres may have significant telomerase activity. Telomerase may therefore be an effective target for anti-tumour drugs.

In most unicellular eukaryotes, telomere length is a dynamic variable with both expansion and contraction events occurring under different culture conditions^{5,7,8}. Complex regulation of telomere length is not unexpected in immortal, single-cell organisms which experience dramatic changes in their environment. However, it is possible that mortal, somatic cells of higher eukaryotes lack mechanisms for telomere elongation. Telomeric sequences may be added exclusively in the germ line, thus explaining the observation that sperm telomeres are longer than telomeres from normal somatic cells^{17,21,29} and these telomeres may simply be lost during the life span of an individual.

There are a number of possible mechanisms for loss of telomeric DNA during ageing, including incomplete replication, degradation of termini (specific or nonspecific), and unequal recombination coupled to selection of cells with shorter telomeres³⁰. Two features of our data are relevant to this question. First, the decrease in mean telomere length is about 50 bp per mean population doubling (Fig. 2a) and, second, the distribution does not change substantially with growth state or cell arrest. These data are most easily explained by incomplete copying of the template strands at their 3' termini. But the absence of detailed information about the mode of replication or degree of recombination at telomeres means that none of these mechanisms can be ruled out. Further research is required to determine the mechanism of telomere shortening in human fibroblasts and its significance to cellular senescence. □

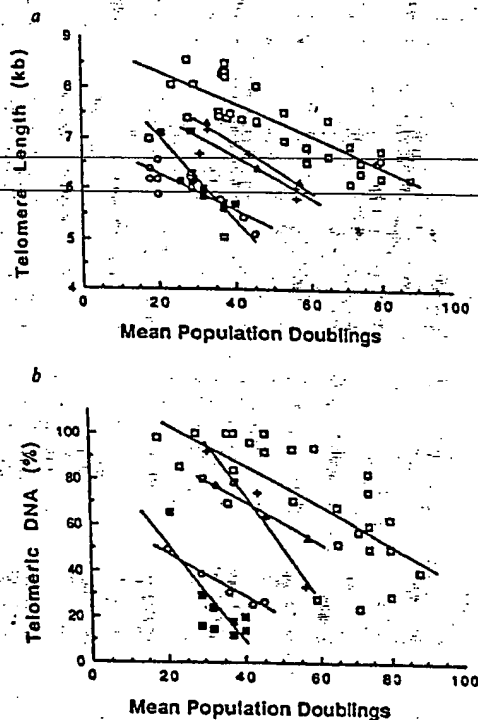


FIG. 2 Decrease in amount of telomeric DNA during replicative ageing of cultured human fibroblasts. Mean telomere length (a) and total telomeric DNA (b) are shown as a function of *in vitro* age. Symbols represent fetal cell strain HSC172 (□), newborn strain A30S (Δ), young adult strain A38 (+), and old adult strains F001 (■) and F002 (○) (see Table 1). The slopes of the linear regression lines are: -31 bp MPD^{-1} , $-0.9\% \text{ MPD}^{-1}$ (HSC172); -48 bp MPD^{-1} , $-0.9\% \text{ MPD}^{-1}$ (A30S); -41 bp MPD^{-1} , $-2.3\% \text{ MPD}^{-1}$ (A38); -85 bp MPD^{-1} , $-2.1\% \text{ MPD}^{-1}$ (F001); and -37 bp MPD^{-1} , $-1.0\% \text{ MPD}^{-1}$ (F002). Analysis of HSC172 cells include data collected from two separate ampules of cells grown at different times.

METHODS. DNA digested with *MspI* and *RsaI* was quantified and 2 μg run in each lane of various 0.7% agarose gels for transfer to Nytran filters and hybridization with (TTAGGG)_n as described in Fig. 1. Autoradiographs were generated without an intensifying screen using the linear response range of the film and scanned with a densitometer. The signals probably include hybridization to variant forms of TTAGGG. Output was digitized. Mean telomere length was defined as $\Sigma(\text{OD})_i / \Sigma(\text{OD})_i / L_i$, where OD_i is the densitometer output (arbitrary units) and L_i is the length of the DNA at position i . Sums were calculated over the range 3–17 kb. This calculation assumes that DNA transfers at equal efficiency from all points in the gel and that the number of target sequences (telomere repeats) per DNA fragment is proportional to DNA length. Although neither of these assumptions is strictly true, the data did not warrant a more complicated model. Qualitatively similar results were obtained with other assumptions and methods. To estimate loss of total telomeric DNA, the integrated signal (ΣOD , from 3–17 kb) was determined for each lane and, where possible, normalized to the signal from other Southern blots using a control probe. Lanes in which amounts other than 2 μg of DNA had been loaded were omitted from the analysis. Integrated signals for each autoradiograph were then expressed as a percentage of the signal from early passage HSC172 DNA on each autoradiograph as a standard.

Received 5 October, 1989; accepted 4 April 1990.

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ACKNOWLEDGEMENTS. We thank R. Allshire and S. Goldstein for helpful discussions, C. Patterson for suggestions for strains F001 and F002, J. Regoeczi for technical assistance, M. Eastwood for HSC172 and S. Goldstein and E. Moerman for DNA from strains A30S, A38 and A35. C.W. and A.E. thank Cold Spring Harbor for support. C.B.H. thanks the Medical Research Council of Canada.

PERSPECTIVES

CHROMOSOME ERROR
PROPAGATION and CANCER

ROBIN HOLLIDAY

The partitioning of the genetic material during the division of normal cells depends on accuracy at several levels. The accuracy of DNA synthesis itself is well known, primarily from many measurements of the rate of gene mutation¹, but the distribution of DNA equally to daughter cells depends in eukaryotic organisms on many coordinated events, which involve the splitting of chromosomes to form chromatids and the separation of these at mitosis to form two cells, each with the exact complement of chromosomes and DNA. The accuracy of disjunction of chromosomes at mitosis can be measured by genetic methods and is generally very high, approximately 1 error in 10^4 mitotic divisions for a given genetically marked chromosome (see below).

A general feature of tumour cells is the loss of the accuracy of chromosome disjunction, leading to aneuploidy and heteroploidy. Thus, a tumour cell population characteristically has a modal number of chromosomes, with considerable variation about this mode. When tumour cells are cloned, they soon generate a population of cells with a variable karyotype. Hence, in these cells nondisjunction is a common event, perhaps two or three orders of magnitude more frequent than in normal diploid cells.

In the genesis of heteroploid tumour cells, the following crucial question can therefore be asked: what event is most likely to convert a cell with a stable karyotype to a cell with an unstable one? In the ensuing discussion, I suggest that rare nondisjunction or rearrangement of at least one specific chromosome may itself be the initial destabilizing event. The argument is based on the proposal of Meeks-Wagner and Hartwell² that gene dosage and the concentration of one or more gene products is a very important component in the accurate control of normal chromosome disjunction.

ACCURACY OF CHROMOSOME DISJUNCTION

The most reliable method for measuring the frequency of mitotic chromosome nondisjunction depends on the use of a diploid strain with heterozygous markers on each side of a single centomere. With a selective system, homozygosity or hemizygosity for all markers can be readily detected, following chromosome loss with or without reduplication of the monosomic chromosome. Several such systems have been developed in yeast and these show that the frequency of spontaneous nondisjunction for a single chromosome is about 10^{-5} per cell division³⁻⁵.

Unfortunately, similar genetic markers are not available in diploid mammalian cells, but naturally occurring heterozygotes have been used to measure the change or loss of one allele, for example, by selection against one of two HLA-A haplotypes on chromosome 6 in T lymphocytes. The clones that are selected may have an altered mutant allele, or may have lost the allele. Loss can occur by deletion, mitotic recombination or nondisjunction. In such experiments the frequency of gene loss is about 3×10^{-4} (Ref. 5 and A.A. Morley, pers. commun.), which means that chromosome loss cannot occur at a higher frequency than this, unless monosomic cells are nonviable.

Most studies of nondisjunction in diploid mammalian cells have relied on counting metaphase chro-

A general characteristic of tumour cells is their unstable karyotype. It is suggested here that maintenance of the normal diploid cell depends on the presence of two copies of specific genes; a change in gene dosage of one or more of these genes, by chromosome nondisjunction or rearrangement, may trigger a general loss of accuracy in chromosome segregation at mitosis.

mosomes, which is probably a very unreliable method. For example, in many studies of the chromosomes of human phytohaemagglutinin-stimulated lymphocytes (reviewed in Ref. 6), hyperdiploid frequencies varied from 0.1 - 4.2% with a mode of 0.4%. The range for rodent lymphocytes was similar, with a mode of 0.5%. If the modes are taken as the best measurement, this indicates that errors in disjunction per individual chromosome are about 0.01% or 10^{-4} . The variability of published results may be due to the loss or gain of chromosomes in the spreading technique. Another cytological method depends on the detection of micronuclei containing whole chromosomes which have become detached from the metaphase plate and are not included in daughter nuclei⁷. These occur in normal human T lymphocytes at a frequency of about 10^{-3} - 10^{-2} (Ref. 8 and A.A. Morley, pers. commun.), corresponding to about 10^{-4} - 10^{-3} per chromosome.

Many tumour cell lines are heteroploid with a continually varying chromosome number. In such cells, there is probably at least one abnormal chromosome segregation per division, and there may be several. The overall frequency of nondisjunction is likely to be at least 100-fold higher than that of normal diploid cells. Some transformed or tumour cells have been reported to have diploid or quasi-diploid karyotypes, but such populations tend to give rise to hypo- or hyper-diploid cells⁹. The pseudo-diploid Chinese hamster ovary (CHO) cell line has been used extensively in somatic cell genetics, and appropriate markers make it possible to measure chromosome nondisjunction. In hybrids heterozygous for two X chromosome-linked markers, abnormal segregation occurred at rates of $1.4 - 3.0 \times 10^{-3}$ per cell division in different experiments¹⁰, which is almost certainly considerably higher than the rate in diploid cells.

GENE DOSAGE IN THE NORMAL CELL CYCLE

The cell cycle includes both continuous and discontinuous events. Total protein synthesis of the cell is a continuous process, so the protein:cell volume ratio stays roughly constant. The duplication of a gene at a particular time in the S phase is a discontinuous event. It is well established from many studies with eukaryotes that the concentration of a given protein is proportional to the number of genes coding for that protein. Thus, when a gene is replicated the amount of transcription is doubled and with a constant rate of mRNA translation, the amount of gene product is also

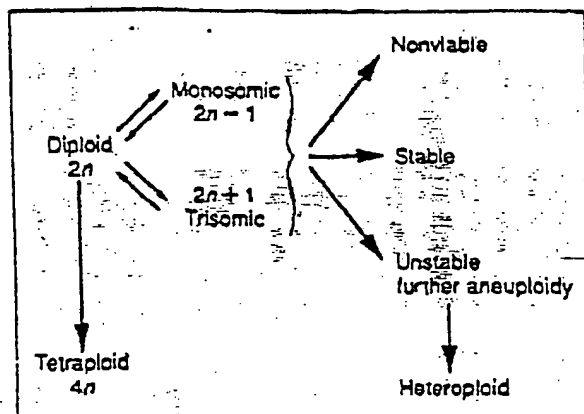


FIG 11

CHROMOSOME CHANGES IN DIPLOID SOMATIC CELLS. NONDISJUNCTION AT MITOSIS GIVES MONOSOMIES (LOSS) OR TRISOMIES (GAIN), WHICH MAY BE NONViable, REVERT TO DIPLOIDY BY SECONDARY NONDISJUNCTION (OR ENDOREDUPPLICATION) OF THE AFFECTED CHROMOSOME, OR PRECIPITATE NONDISJUNCTION OF OTHER CHROMOSOMES. IN PARTICULAR, IT IS PROPOSED THAT GENES CONTROLLING THE ACCURACY OF MITOTIC SEPARATION OF ALL CHROMOSOMES WILL HAVE LESS ACCURATE CONTROL IF PRESENT IN ONE OR THREE COPIES. THUS, NONDISJUNCTION OF A CHROMOSOME WITH SUCH A GENE WILL LEAD TO FURTHER CHROMOSOME CHANGES AND A GENERAL DESTABILIZATION OF THE KARYOTYPE.

doubled. Therefore the ratio of this protein to cell volume will change through the cell cycle over roughly a twofold range, although other cell cycle controls may well be superimposed on rates of transcription and translation.

The most detailed experimental studies of the cell cycle have been carried out in bacteria and yeasts. A general conclusion is that a doubling of the concentration of one or more specific proteins in the cell is likely to be an important component in controlling the progress of any cell through the cycle. Evidence for this comes from the study of mutations that change cell volume and from the experimental manipulation of growth conditions that change the initiation and/or the rate of DNA synthesis, as well as cell volume¹¹⁻¹³.

One of the events of the cell cycle that could be critically dependent on gene dosage is the accurate disjunction of chromosomes². The replication of one or more critical genes would raise the level of gene product to that which is optimal for accurate centromere separation and the normal function of the mitotic spindle fibre apparatus. But suppose that the chromosome containing one of these critical genes undergoes spontaneous nondisjunction. The daughter cells following mitosis will now have one or three copies of the gene in question. Thus, in the next cell cycle there will be over- or under-expression of the important gene product, and this will increase the probability of errors in chromosome disjunction at the next and subsequent mitoses. This is an example of error propagation, in which one or more initial errors feed back to make later events even less accurate.

Although histones are unlikely to have a direct regulatory role in the control of mitosis itself, it is significant that overexpression of histone genes leads to a significant increase in mitotic nondisjunction in yeast⁴. Overexpression of one set of genes near the G1-S boundary in the cell cycle may well be correlated with

a change in expression of other genes that are normally tightly regulated.

Random nondisjunction of individual chromosomes can lead to several possible consequences (Fig. 11). The initial unbalanced karyotype may be nonviable, or result in slow growth, followed by selection for cells that regain the diploid karyotype by endoreduplication or a second nondisjunction event. Monosomy or trisomy for small chromosomes may be stable. The particular consequence proposed here is that destabilization of the genome will be triggered by nondisjunction of a chromosome carrying a gene that must be optimally expressed to ensure the accuracy of subsequent mitoses. This event is therefore likely to increase the frequency of nondisjunction, which may affect a second chromosome carrying a gene with a similar regulatory role. Return to the diploid karyotype then becomes extremely unlikely and the cell either dies or becomes irreversibly committed to further aneuploidy. An unbalanced genome produced by an initial chromosome rearrangement could produce the same end effect. The basic principle is that initial abnormalities in chromosomes containing genes that are essential for the control of disjunction will destabilize the karyotype. The upshot would be the situation that is usual in tumour cells—namely, continuous variation in chromosome number about a modal number.

Previous discussions of error propagation have dealt mainly with the possibility that errors in proteins required for information transfer may destabilize the accuracy of protein synthesis itself, leading to an ever-increasing number of errors^{14,15}. The same principle can be applied to chromosome segregation. Initial errors ultimately lead to a 'chromosome catastrophe', in which there is uncontrolled karyotypic variability.

GENE DOSAGE AND CHROMOSOME CHANGES IN CARCINOGENESIS

The importance of chromosome changes in carcinogenesis has been widely recognized (reviewed in Refs 9, 16-19). More specifically, the significance of changes in gene dosage has been emphasized by Klein²⁰. In many instances, cellular proto-oncogenes are activated to oncogenes by being inserted into new genetic locations where transcription is increased. Although some oncogenes arise by mutation to an activated form, in many cases it seems likely that mere overexpression of the normal gene product is a critical step in carcinogenesis^{20,21}.

Innumerable studies of karyotypic changes in human cancer cells have been published and the results catalogued in comprehensive surveys^{22,23}. A major problem in this area of investigation is to distinguish primary from secondary events; although a progression of chromosome changes can sometimes be discerned in the growth of tumour clones¹⁷, the significance of the earliest detectable chromosome changes is hard to evaluate. Heim and Mitelman¹⁹ suggest that chromosome aberrations in neoplastic disorders are of three kinds: (1) primary abnormalities, which are essential steps in establishing the tumour, (2) secondary abnormalities, which may be important in tumour progression, and (3) 'cytogenetic noise', which is the background level of nonconsequential aberrations.

Holliday

Whatever the primary cause, it is nevertheless probable that destabilization of the genome is a very important early event in tumour progression. Following that destabilization, a wide variety of abnormal genotypes and phenotypes will rapidly be produced and successive clonal selection for rapid growth, tumorigenicity and metastasis will therefore follow. As a result of karyotypic instability, tumour suppressor genes (reviewed in Refs 24, 25) may become hemizygous; this will make them targets for a single recessive mutation, whereas in diploid cells at least two rare events are required to eliminate suppressor gene activity. During clonal selection for cancer cells it is possible that a partial reversal of instability may be favoured, if a cell with a particular karyotype is the most invasive. It should be noted that although the many karyotypic studies of neoplastic human cells show that chromosome changes are not random^{22,23}, they are not likely to reveal those chromosomes that control the accuracy of disjunction, since aneuploidy for one or more of these chromosomes may rapidly lead to other chromosome changes that obscure the initial event.

It is well known that agents that induce karyotypic changes can lead to transformation. For example, the oncogenic simian virus 40 (SV40) rapidly destabilizes the genome^{26,27}. Colcemid and diethylstilbestrol produce aneuploidy, and they can also induce neoplastic transformation in primary Syrian hamster cells^{28,29}. Chemically inert agents such as asbestos and glass fibres can also induce transformation and chromosome changes in these cells³¹. The carcinogenic activity of asbestos is striking, and it may be that cellular ingestion mechanically disrupts normal spindle function, which leads to aneuploidy. Most carcinogens induce chromosome changes and it can be argued that the correlation between these two effects is greater than that between mutagenicity and carcinogenicity^{16,18}.

INTRINSIC GENOME INSTABILITY OF TRANSFORMED CELLS

Although the loss of accuracy of chromosome disjunction in cancer cells is a major component of karyotypic instability, it is by no means the only one. Chromosome translocations and other rearrangements are clearly more common in cancer cells than in normal cells. Several rearrangements are commonly seen in the karyotype of a single cancer cell, whereas even a single rearrangement is uncommon in a normal diploid population. In addition, cancer cells often amplify regions of DNA to produce drug-resistant derivatives, or they may have amplified oncogenes³². Experimental studies on amplification have been carried out with transformed or partially transformed mammalian cells^{33,34}, but there are no published reports of normal mammalian diploid cells becoming resistant to toxic drugs or metabolites by amplifying specific genes. This suggests that the linear integrity of the DNA in chromosomes of diploid cells is much more strictly controlled than it is in transformed cells.

In recent years, the transfection of mammalian cells by DNA has become a standard procedure. Methods for promoting the uptake of DNA have been developed, and this DNA often integrates at nonhomologous regions of the chromosome. However, almost all

TABLE 1. MAJOR DIFFERENCES BETWEEN THE GENETIC CHARACTERISTICS OF NORMAL DIPLOID MAMMALIAN SOMATIC CELLS AND TRANSFORMED CELLS (SEE TEXT FOR REFERENCES)

	Normal cells ^a	Transformed cells ^b
Karyotype	Diploid ^c	Aneuploid or heteroploid
Nondisjunction of chromosomes	Low frequency	High frequency
Chromosome rearrangements	Uncommon	Common
Gene amplification	Not reported	Well documented
Integration of foreign DNA	Very rare ^d	Readily detected
Oncogenes	Normal proto-oncogenes	Activated or over-expressed oncogenes
DNA methylation	Declines during subculture	Constant with <i>de novo</i> methylation

^aSomatic diploid cells with finite division potential.

^bIncludes partially transformed permanent lines and fully transformed tumorigenic cells.

^cTetraploid cells which occur in some tissues and primary cell cultures also have a balanced genome and appear to be stable.

^dShort term assays, using plasmids with the chloramphenicol acetyltransferase gene, show that DNA is taken up by somatic diploid cells, but several laboratories have failed to obtain stable transfectants with DNA integrated into a chromosome. (For obvious reasons, these negative results remain unpublished.) An illustration of this comes from a comparison of human diploid fibroblasts, strain MRC-5, and its SV40-transformed derivative. Both cells take up exogenous DNA, but stable transfection is very rare in the diploid parent, whereas it is frequent in the transformed derivative (L.I. Huschtscha, pers. commun.). However, DNA can be integrated in the chromosomes of eggs or embryonic cells, and into somatic cells using retrovirus vectors.

the experiments in which DNA is integrated have been carried out with transformed cells, permanent lines or embryonic cells. Uptake of DNA can be readily demonstrated in normal somatic diploid cells, but integration into the chromosome is probably a rare event (see footnote d, Table 1), unless, of course, it is mediated by a retrovirus. Again, these cells appear rigorously to maintain the stability of a normal genome.

There may also be important differences in the postsynthetic modification of DNA in normal and transformed cells. Diploid cells have finite proliferative potential and during serial subculture the level of 5-methylcytosine (m⁵C) in their DNA progressively declines, whereas permanent lines and transformed cells maintain a constant level of m⁵C (Refs 35, 36, F. Malik and R. Holliday, unpublished). Although this implies that DNA methylation is at a steady state in these cells, it is probable that the overall control of DNA methylation is abnormal (see Ref. 37). Permanent lines often have silent genes that can be reactivated by 5-azacytidine, strongly indicating that the lack of gene expression is due to DNA methylation. The published evidence suggests that such cells frequently inactivate genes by *de novo* methylation.

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On the other hand, the phenotype of diploid cells is strictly conserved, presumably by the tight control of expression of luxury genes and the concomitant lack of expression of specialized gene functions of other cell types. It is therefore possible that a general destabilization of the genome may also include a loss of the normal epigenetic controls of gene expression, which may in turn be important steps in tumour progression. The major differences in the genetic stability of normal and transformed cells are listed in Table 1.

CONCLUSION

The multistep nature of carcinogenesis is well established, but the genetic changes associated with the various stages of tumour progression are usually ill defined. They may include gene mutation, recombination, gene amplification, chromosome abnormalities, DNA transposition or epigenetic changes in gene expression (reviewed in Ref. 39). A crucial step in tumour progression appears to be a destabilization of the genome: this leads to the production of aneuploid or heteroploid cells and probably the loss of other controls that are necessary to preserve the integrity of the genome. Mutation of hemizygous tumour suppressor genes may also be an important component of the overall genetic diversity that allows the sequential selection of clones of cells with progressively more invasive and metastatic phenotypes. Several inherited conditions in man are associated with increased chromosome abnormality and predisposition to cancer³⁹.

The fundamental difference between the genetic stability of normal cells and the instability of tumour cells is very striking, and suggests that an important early event in carcinogenesis is the loss of accuracy in chromosome segregation or disjunction at mitosis. This accuracy may depend on diploid gene dosage for one or a few critical genes, and thus an alteration in this dosage may reduce the accuracy of disjunction. If this is correct, then nondisjunction for a chromosome carrying one of the critical genes will increase or decrease this gene dosage, which will then increase the likelihood of subsequent changes in chromosome number. This would be an example of error propagation in which a single defect will ultimately by feed-back give rise to many further errors.

There are two reasons why changes in gene dosage may be important in the regulation of cell growth. First, the cellular concentrations of critical gene products appear to be very important in the control of the normal cycle, and the accurate partitioning of genetic material in mitosis is one component of that control. Second, overexpression of proto-oncogenes may be an important step in cellular transformation, and this may also be related to changes in karyotype.

Loss of control of chromosome number is also probably associated with an increase in other genetic alterations, such as chromosome rearrangement, gene amplification, integration of foreign DNA, and abnormalities in DNA methylation. In this way the variability and potential for evolution of fully tumorigenic cells is greatly increased. If this view is correct, then an understanding of the processes that bring about the loss of genetic stability and carcinogenesis will also

depend on an understanding of the mechanisms that accurately maintain the integrity of the genome of somatic cells through successive cell divisions.

ACKNOWLEDGEMENTS

I thank Felix Mitelman, Alec Morley, Paul Nurse, Robert Rosenberger and Geoffrey Grigg for their helpful comments.

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Expression of oncomodulin does not lead to the transformation or immortalization of mammalian cells *in vitro*

COPY AS FILED

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Summary

A recombinant plasmid (pMTONCO) containing the coding sequences for rat oncomodulin under the direction of the metallothionein promoter was constructed. pMTONCO was co-transfected with the pSV2-NEO plasmid into primary mouse kidney cells or Rat-1 cells using the calcium phosphate technique and stable transformants were isolated after selection with G418. Transcription from the metallothionein promoter was inducible with heavy metals and produced an oncomodulin-specific mRNA. The presence of oncomodulin protein in stable cell lines was verified by immunoprecipitation with specific antisera. While a plasmid encoding the polyomavirus T-antigens was

able to prolong the life-span of primary mouse kidney cells in culture, no equivalent activity was noted when the pMTONCO plasmid was used to transfect primary cells. When expressed in Rat-1 cells, oncomodulin did not affect the growth properties of these cells, nor did it predispose cells to higher frequencies of oncogenic transformation to a viral oncogene. We conclude that oncomodulin is neither an immortalizing nor transforming agent *in vitro*.

Key words: oncomodulin, oncodevelopmental protein, transformation.

Introduction

Oncomodulin was initially identified as a novel low molecular weight calcium-binding protein found in extracts of rat liver tumors (MacManus, 1979). Direct protein sequencing has demonstrated the sequence of the 108 amino acids that comprise the complete protein (MacManus *et al.* 1983). Recently, the amino acid sequence of rat oncomodulin has been confirmed, with the exception of a single amino acid difference, by sequence analysis of a complete cDNA encoding this calcium-binding protein (Gillen *et al.* 1987). These results, in addition to earlier immunological evidence (MacManus, 1981a), clearly demonstrate that oncomodulin is distinct from previously described calcium-binding proteins, but shares sufficient homology to be considered a member of the troponin C superfamily (MacManus *et al.* 1983). Oncomodulin shares the greatest homology with rat parvalbumin, at both the nucleic acid and the amino acid level (Gillen *et al.* 1987; MacManus *et al.* 1983).

In rats, oncomodulin is normally expressed extra-embryonically in placenta, yolk sac and amnion (Brewer and MacManus, 1985). While oncomodulin has never been detected in normal adult rat tissue, interest in this protein stems mainly from the finding that oncomodulin

is present in tumor tissue of rat hepatomas (MacManus, 1979), rat fibrosarcomas and mouse sarcomas (MacManus, 1981a). In addition to its presence in spontaneously occurring rodent tumors, oncomodulin has also been detected in solid tumors following the injection of rodent transformed cell lines into nude mice (MacManus *et al.* 1982). The synthesis of oncomodulin has also been observed in the nucleus of virally transformed normal rat kidney cells *in vitro* (Durkin *et al.* 1983). The presence of a calcium-binding protein similar to rodent oncomodulin has also been observed in human tumors (MacManus and Whitfield, 1983; MacManus *et al.* 1984), cell lines derived from human tumors (Pfyffer *et al.* 1984), or tumors formed in nude mice following injection of transformed human cell lines (MacManus *et al.* 1982). Recent results indicate that the expression of oncomodulin in rat tumor tissue is regulated at the level of mRNA transcription (Gillen *et al.* 1987).

The role of oncomodulin, as well as its possible function within the tumor cell, remains unclear. While it has been demonstrated that oncomodulin has a calmodulin-like ability to stimulate the hydrolysis of cyclic AMP by rat heart phosphodiesterase (MacManus, 1981b; Mutus *et al.* 1985), and to a much lesser extent rat brain phosphodiesterase (Klee and Heppel, 1984), oncomodulin does not generally mimic the activity of calmodulin.

Although oncomodulin has been shown to stimulate calcium-starved non-neoplastic liver cell DNA synthesis (Boynton *et al.* 1982), the interpretation of this result is difficult, since oncomodulin is not present in regenerating liver (MacManus and Whitfield, 1983).

Like alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA), oncomodulin appears in both developing and neoplastic tissues, and as such is considered an oncodevelopmental protein (Brewer and MacManus, 1985). While oncodevelopmental proteins may be useful as tumor markers, their role, if any, in the development or progression of carcinogenesis remains unknown. In contrast, oncogenes clearly play a role in neoplasia. At least two discernable activities have been described for oncogenes *in vitro*: their ability to immortalize primary cells and/or to transform established cell lines in culture (Rassoulzadegan *et al.* 1982; Land *et al.* 1983; Ruley, 1983). Because oncomodulin appears to be almost ubiquitously present in rodent tumor tissues (MacManus, 1979; MacManus, 1981a; MacManus *et al.* 1982), and since it has been shown to influence DNA synthesis indirectly (Boynton *et al.* 1982), it was of interest to determine whether the expression of oncomodulin could influence

the morphology and growth patterns of cells in a manner similar to oncogenes. A recombinant clone permitting the expression of rat oncomodulin in mammalian cells was constructed and introduced into primary baby mouse kidney cells or cells from the established Rat-1 line by DNA transfection. Our results indicate that oncomodulin does not act as a cellular immortalizing or transforming agent *in vitro*.

Materials and methods

Recombinant plasmids

All enzymatic reactions were performed as described by the manufacturer. Standard protocols for plasmid DNA purification, agarose gel electrophoresis, DNA fragment purification and plasmid transfection into bacteria were employed (Maniatis *et al.* 1982).

Details of the construction of the pMTONCO plasmid are described in Fig. 1. The starting plasmid, pPXMT, was originally provided by J. Sambrook (University of Texas Southwestern Medical Center) and was obtained from J. A. Hassell (McMaster University). Apart from plasmid sequences, pPXMT contains a 0.6 kb (1 kb = 10^3 bases) KpnI-BglII fragment of the mouse metallothionein I promoter and a

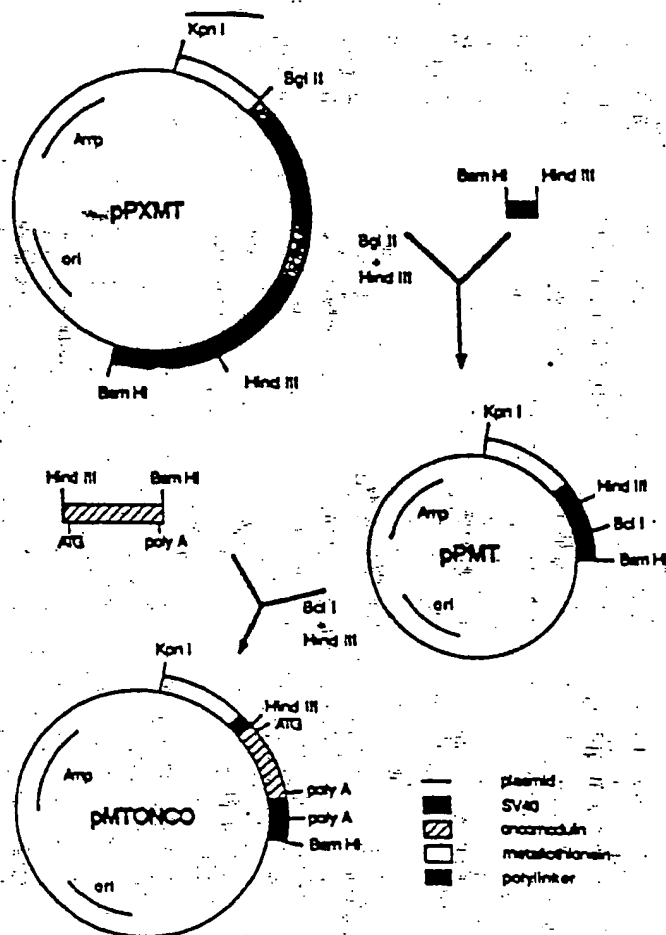


Fig. 1. Schematic representation of the cloning steps involved in the construction of the recombinant plasmid pMTONCO.

*Bgl*II (formally a *Bgl*I site) - *Bam*HI fragment (spanning nucleotides 5235-2533) of the simian virus (SV40) early region. pPXMT was digested with *Bgl*II and *Hind*III to remove the majority of the SV40 early region, leaving only SV40 sequences from the *Hind*III-*Bam*HI site (nucleotides 3476-2533), which contain, among other sequences, the early SV40 polyadenylation signal. The digested fragment was ligated to a small *Bam*HI-*Hind*III polylinker fragment obtained from the plasmid pGEM-1 (Promega). The resulting recombinant (pPMT) was purified from *Escherichia coli* GM150, a *dam* methylase minus strain. pPMT DNA was cleaved by restriction endonuclease digestion, and the large *Hind*III-*Bcl*I fragment was gel purified. In parallel, a cDNA fragment of rat oncomodulin, containing a *Hind*III linker at nucleotide +10 and a *Bam*HI linker past nucleotide +660 (Gillen *et al.* 1988) was gel purified and ligated to the large *Hind*III-*Bcl*I fragment of pPMT. The resulting plasmid, pMTONCO, was partially sequenced to ensure that no alterations had occurred in sequences surrounding the AUG translation initiation codon of oncomodulin.

Two other recombinant plasmids were also employed in this study. pSV2NEO has previously been described (Southern and Berg, 1982). The plasmid pSV2NEOSVEB1a was a kind gift from Bernard Massie (Biotechnology Research Institute, National Research Council of Canada). Briefly, pSV2NEOSVEB1a contains both the large *Bam*HI-*Pvu*I fragments of pSV2NEO and pPSVE1-B1a (Muller *et al.* 1984), so that the synthesis of neomycin or the synthesis of small, middle and large T-antigens of polyomavirus are driven by separate SV40 early promoter regions.

Cell culture and transformation

All cells were propagated in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, gentamicin ($50 \mu\text{g ml}^{-1}$) and Fungizone ($2.5 \mu\text{g ml}^{-1}$). Cells were maintained at 37°C in a humidified CO_2 incubator. Rat-1 cells, a subclone of Fischer rat F2408 cells (Freeman *et al.* 1973) were obtained from J.A. Hassell (McMaster University). Primary mouse baby kidney (BMK) cells were prepared as described previously (Freshney, 1987).

Cesium chloride gradient-purified supercoiled plasmid DNA was used to transfect Rat-1 or BMK cells. Rat-1 and BMK cells were seeded onto 100 mm plastic dishes at a cell density 3.5×10^5 cells/plate and 5×10^5 cells/plate, respectively. Fifteen hours post-plating, one ml of calcium phosphate-precipitated DNA (Wigler *et al.* 1978) containing a total of $20 \mu\text{g}$ of DNA (consisting of plasmid DNA and calf thymus DNA if needed) was added per 100 mm dish. The precipitate was allowed to settle on the Rat-1 cells (4 h) or the BMK cells (overnight), after which the precipitate was washed off with phosphate-buffered saline and fresh medium was added. At 48 h post-transfection, cells were put under G418 selection ($400 \mu\text{g ml}^{-1}$ for Rat-1 cells, $120 \mu\text{g ml}^{-1}$ for BMK cells). G418-resistant colonies were cloned 14 to 21 days post-transfection by lifting colonies with trypsin-impregnated Whatman paper, which was then deposited in 24-cluster microwell dishes containing G418-supplemented media.

To establish cell growth patterns, cells were initially plated at 1×10^5 cells per 60 mm plate. Sufficient plates were established such that duplicate 60 mm plates of cells were trypsinized and counted at each time point. To test the capacity of cells to grow without a solid support, 1×10^5 cells were suspended in 0.33% (w/v) agarose in DMEM containing 10% fetal bovine serum and plated over a layer of 0.60% (w/v) agarose in the same medium. After one week, the plates were scored for the presence of colonies.

Northern blot analysis

RNA was extracted from cells as previously described (Chirgwin *et al.* 1979). Since the metallothionein promoter is responsive to heavy metal induction, some cells were maintained for 2 h in metal-supplemented medium (10 mM-zinc chloride, 1 mM-cadmium chloride) before RNA extraction. Total RNA ($10 \mu\text{g}$) was electrophoretically separated in a denaturing agarose/formaldehyde gel, the gel was stained with ethidium bromide to verify the integrity and concentration of the RNA sample, and the RNA was transferred to nitrocellulose using standard techniques (Maniatis *et al.* 1982). Rat oncomodulin sequences were detected by hybridization to a [^{32}P]CTP-labelled RNA probe complementary to the coding sequences of oncomodulin mRNA sequences. Hybridizations were performed at 60°C in 50% formamide, 50 mM-sodium phosphate (pH 7.0), 800 mM-sodium chloride, 1 mM-EDTA (pH 8.5), 2.5 \times Denhardt's solution, 250 $\mu\text{g ml}^{-1}$ denatured salmon sperm DNA and 500 $\mu\text{g ml}^{-1}$ yeast tRNA. Hybridization mixtures also contained radioactively labelled RNA at 2×10^6 cts $\text{min}^{-1} \text{ml}^{-1}$. Excess probe was removed after hybridization by three successive 30-min washes at 60°C in 50 mM-sodium chloride, 20 mM-sodium phosphate (pH 7.0), 1 mM-EDTA (pH 8.5) and 0.1% (w/v) sodium dodecyl sulfate (SDS). Dried filters were exposed, with intensifying screens, at -80°C for 1-5 days with Kodak XAR-3 film.

Analysis of oncomodulin protein

Newly synthesized proteins were metabolically labelled by incubating 8×10^5 cells/60 mm dish in 0.5 ml methionine-free DMEM, containing 100 μCi of [^{35}S]methionine (800 Ci mmol^{-1}). Immunoprecipitation of labelled oncomodulin protein was performed as previously described (Chalfour *et al.* 1986). Affinity-purified goat anti-oncomodulin IgG was kindly provided by J.P. MacManus (Division of Biological Sciences, National Research Council of Canada). Immunoprecipitated proteins were separated by electrophoresis in a 15% SDS-polyacrylamide gel (Laemmli, 1970). After gel fluorography with Enhance (New England Nuclear), the gel was dried and the labelled immunoprecipitated proteins were visualized by exposing the gel for 14 days with XAR-5 Kodak film.

A quantitative analysis of oncomodulin protein levels was performed by radioimmunoassay using a previously described protocol (MacManus *et al.* 1982).

Results

Expression of oncomodulin in Rat-1 cells

In order to assess directly the effect of oncomodulin on cellular transformation, the recombinant plasmid pMTONCO was introduced into an established rat fibroblast cell line (Rat-1) in which no expression of the endogenous oncomodulin gene was detected. Rat-1 cells were transfected with either 50 ng of pSV2NEO per 100 mm dish or co-transfected with a mixture of 50 ng pSV2NEO and 250 ng pMTONCO per 100 mm dish. After selection, well-isolated G-418-resistant colonies were cloned. A G-418-resistant Rat-1 cell line that had been transfected with pSV2NEO DNA was isolated and this cell line, NEO5, was used as a negative control throughout this work. Nine G-418-resistant colonies were cloned following transfection of the Rat-1 cell line with precipitates containing both the pSV2-neo and pMTONCO plasmids.

Total RNA was isolated from either control (RAT-1

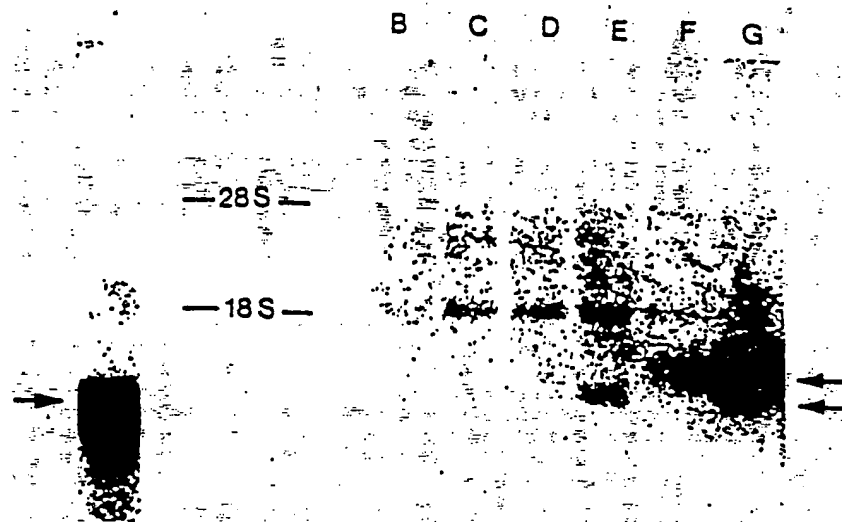


Fig. 2. Northern blot analysis of RNA from either control or cell lines expressing the rat oncomodulin cDNA. Lane A contains RNA purified from rat hepatomas, and the arrow indicates the position at which endogenous rat oncomodulin migrates within a 1.4% agarose/formaldehyde gel. Lanes B and C represent RNA isolated from Rat-1 and NEO5 cells, respectively. RNA was isolated from the MTONCO2-7 cell line either before (lane D) or after (lane E) cells were induced with heavy metals. In a similar manner, RNA from the MTONCO2-12 cell line was isolated before (lane F) or after (lane G) heavy metal induction. Note that the oncomodulin cross-hybridizing species in lanes D, E, F and G do not migrate with endogenous oncomodulin RNA. In addition, the size of the oncomodulin RNA produced by the MTONCO2-7 and MTONCO2-12 cell lines appears to differ by approximately 50–100 nucleotides.

and NEO5) cell lines, or from the nine G-418 cell lines isolated from co-transfection experiments. The latter cell lines were grown in either normal or heavy metal-supplemented media. Of the original nine cell lines, five G-418 cell lines were found to produce detectable levels of oncomodulin mRNA, and in each case the synthesis of oncomodulin RNA was inducible by heavy metals (data not included). Of these five cell lines, two (MTONCO2-7 and MTONCO2-12), producing different constitutive levels of oncomodulin mRNA, were chosen for further study. The oncomodulin RNA pattern of expression of MTONCO2-7 and MTONCO2-12 is shown in Fig. 2. The presence of oncomodulin mRNA can be seen in each case before heavy metal induction, but the oncomodulin-specific mRNA levels increased approximately threefold after induction. The constitutive and induced levels of oncomodulin RNA appear to be higher in the MTONCO2-12 cell line relative to the MTONCO2-7 cell line. The length of the RNA produced in the MTONCO2-7 and MTONCO2-12 cell lines appears to differ by about 50–100 nucleotides.

To determine whether the RNA produced in these cell lines could be translated to produce authentic oncomodulin protein, [35 S]methionine-labelled proteins from control (Rat-1 and NEO5), MTONCO2-7 and MTONCO2-12 cell lines were immunoprecipitated with anti-oncomodulin antisera. An autoradiograph of the immunoprecipitated proteins, after separation by SDS-polyacrylamide gel electrophoresis and fluorography is presented in Fig. 3. Although all cell lines appear to have a 14 000 M_r ,

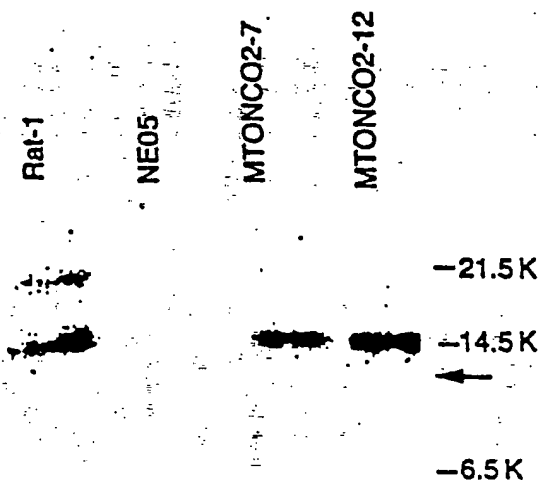


Fig. 3. Autoradiograph of immunoprecipitated 35 S-labelled protein with goat anti-oncomodulin antibody. Control cell lines include the parental (Rat-1) and pSV2NEO-transformed cell line (NEO5). Although all cell lines appear to contain a 14K ($K=10^3 M_r$) background protein, only the MTONCO2-7 and MTONCO2-12 cell lines show a specific 12.6K protein band that correlates with the expected molecular weight for authentic rat oncomodulin.

background band that is precipitated by the anti-oncomodulin antisera, only the MTONCO2-7 and MTONCO2-12 cell lines contain a specifically immunoprecipitated protein of the correct molecular weight for oncomodulin. In addition, radioimmunoassays for oncomodulin were performed. While the Rat-1 and NEO5 cell lines contained undetectable levels of oncomodulin (<25 ng oncomodulin mg^{-1} total protein) both the MTONCO2-7 and MTONCO2-12 cell lines produced significant amounts of oncomodulin. In particular, in the absence of heavy metal stimulation of the metallothionein promoter, the MTONCO2-12 cell line produced upwards of 1500 ng oncomodulin mg^{-1} total protein. This level of expression is comparable to previously reported results for the oncomodulin levels seen in transformed rodent and human cell lines *in vitro* (MacManus *et al.* 1982).

To characterize the influence of oncomodulin expression on patterns of cell growth, the MTONCO2-7 and MTONCO2-12 cell lines were assessed for their growth rate in culture, the ability of cells to form colonies in soft agarose and the ability of cells to form dense foci at confluence. For comparison, the growth patterns of the Rat-1, NEO5 and Rat-1SMLT cell lines were also evaluated. Rat-1SMLT is a polyomavirus-transformed Rat-1 cell line derivative that expresses all three T-antigens, which was isolated following the introduction of the pSV2NEOSVEB1a plasmid into the Rat-1 cell line. The histogram in Fig. 4 indicates the growth rate of the various cell lines. Both the MTONCO2-7 and MTONCO2-12 have growth rates comparable to the parental and pSV2NEO-containing cell lines, while the transformed Rat-1SMLT cell line displays a faster growth rate and is capable of attaining higher cell densities. In addition, the ability of oncomodulin-producing cell lines to grow without solid support was determined. Cells were seeded in soft agarose and allowed to grow for a period of 7-10 days. As shown in Fig. 5, only the polyomavirus-transformed cell line Rat-1SMLT was able to grow without solid support, and

neither the MTONCO2-7 nor MTONCO2-12 cell lines displayed significant growth in soft agarose. Finally, while the Rat-1SMLT cell line did not display contact inhibition and readily formed foci as cells reached confluence, the MTONCO2-7 and MTONCO2-12 cell lines resembled the parental Rat-1 cell line in their inability to form foci, even in heavy metal-supplemented media.

We also wished to address the question of whether the expression of oncomodulin, while not by itself a transforming agent, could predispose a cell to oncogenic transformation. For this purpose the frequency at which different cell lines could be transformed by the plasmid pSV2NEOSVEB1a was assessed and the results presented in Table 1. The transformation frequency is not linear with DNA concentration, since such a linear relationship occurs only at low DNA concentrations (<20 ng), and it has previously been shown that as the amount of DNA becomes saturating the specific trans-

Table 1. Effect of oncomodulin expression on the frequency of neoplastic transformation

Cell line	Transformation frequency*	DNA concentration† (μg)
Rat-1	2075	0.1
	2195	0.05
	3250	0.01
NEO5	560	0.1
	760	0.05
	1300	0.01
MTONCO2-7	700	0.1
	580	0.05
	750	0.01
MTONCO2-12	695	0.1
	710	0.05
	1900	0.01

* Calculated as the total number of foci per μg of pSV2NEOSVEB1a DNA.

† Concentration of pSV2NEOSVEB1a DNA per 100 mm dish.

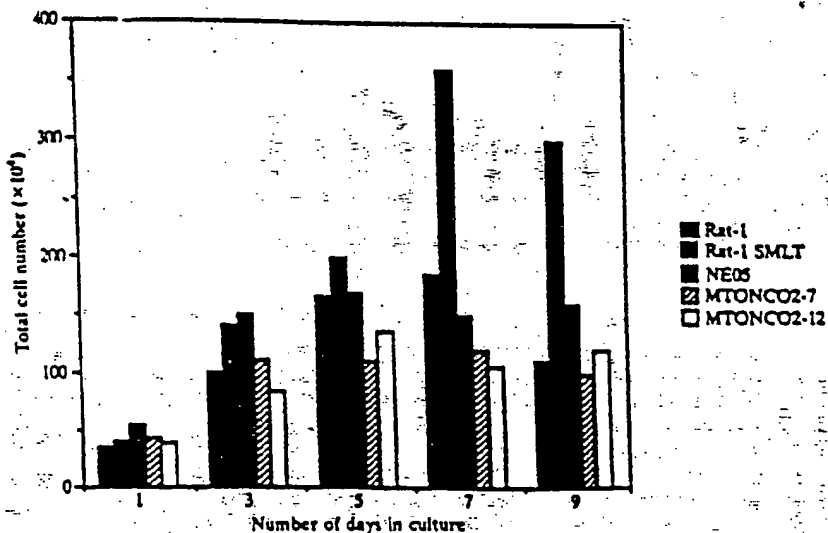


Fig. 4. Histogram representing the growth rate of cell lines in culture. Included are the growth of the parental Rat-1 cell line, a pSV2NEOSVEB1a-transformed cell line (Rat-1SMLT), a pSV2NEO transfected cell line (NEO5), and two independently isolated MTONCO/pSV2NEO co-transfected cell lines (MTONCO2-7 and MTONCO2-12).

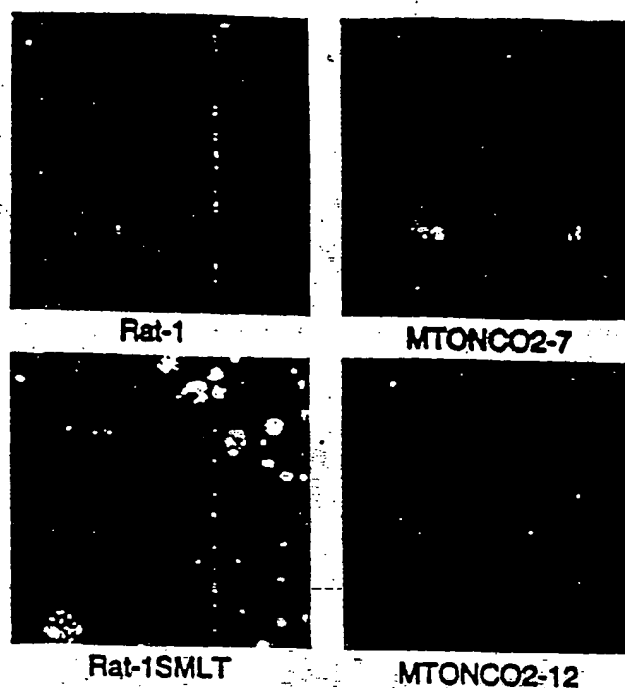


Fig. 5. Growth of cells in soft agarose. Only the polyomavirus-transformed cell line (Rat-1SMLT) was able to form colonies in a semi-solid environment. Neither the parental cell line (Rat-1) nor the oncomodulin-expressing cell lines (MTONCO2-7 and MTONCO2-12) displayed appreciable growth in soft agarose. Cells were photographed through a dissecting microscope at a magnification of $\times 15$.

forming activity declines until a plateau is reached (Mes and Hassell, 1982). Nonetheless, the expression of oncomodulin does not appear to potentiate cellular transformation, as the MTONCO2-7 and MTONCO2-12 cell lines displayed approximately the same frequency of transformation after transfection with pSV2NEOSVEB1a DNA as the NEOS control. By comparing their rate of growth in culture and their ability to form colonies in soft agarose, pSV2NEOSVEB1a-transformed derivatives of both the MTONCO2-7 and MTONCO2-12 cell lines displayed growth patterns indistinguishable from pSV2NEOSVEB1a-transformed Rat-1 cells.

Expression of oncomodulin in primary cells

Some oncogenes, while unable to transform established cell lines in culture, are capable of indefinitely prolonging the lifespan of primary cells in culture and have subsequently been referred to as immortalizing genes (Rasoulzadegan *et al.* 1982; Land *et al.* 1983; Ruley, 1983). An example of this type of immortalizing gene is the large T-antigen of polyomavirus. In order to assess whether the expression of oncomodulin could confer an immortal phenotype on primary cells, 10 μ g of pMTONCO plasmid was co-transfected with 10 μ g of pSV2NEO plasmid per 100 mm dish of BMK cells. As controls, both pSV2NEO and pSV2NEOSVEB1a were independently transfected onto BMK cells at a concentration of 10 μ g of plasmid DNA per 100 mm plate. In all cases cells were put under G418 selection and G418-resistant colonies were cloned. The survival rate of individual colonies is presented in Table 2. Greater than 50% of primary cells isolated after transfection with the pSV2NEOSVEB1a

Table 2. Effect of oncomodulin expression on the ability to clone and propagate BMK cells in culture

Transforming DNA ^a	Cell lines established/ G418-resistant clones picked		
	1	Experiment 2	3
pSV2NEOSVEB1a	4/6	2/3	5/9
pSV2NEOSVEB1a + pMTONCO	2/2	1/3	6/9
pSV2NEO + pMTONCO	0/40	0/12	0/16
pSV2NEO	0/40	—	—

^a DNA used to transfect BMK cells.

plasmid could sustain continued growth in culture, while no cell lines could be established after transfection of primary cells with pMTONCO DNA alone.

To ensure that the oncomodulin mRNA could be produced in primary cells, the pMTONCO and pSV2NEOSVEB1a plasmids were co-transfected onto BMK cells and three G418-resistant cell lines were established. RNA was extracted from these cell lines, separated by gel electrophoresis and probed for the presence of oncomodulin-specific RNA sequences. The results are presented in Fig. 6. While all three cell lines produced polyomavirus-specific RNA (data not included), at least one cell line also expressed RNA specific to oncomodulin. Thus, we conclude that the pMTONCO construct is expressed in primary cells. Since there appears to be no impediment to oncomodulin expression in primary cells, the inability to isolate cell lines after transfection of the pMTONCO plasmid onto

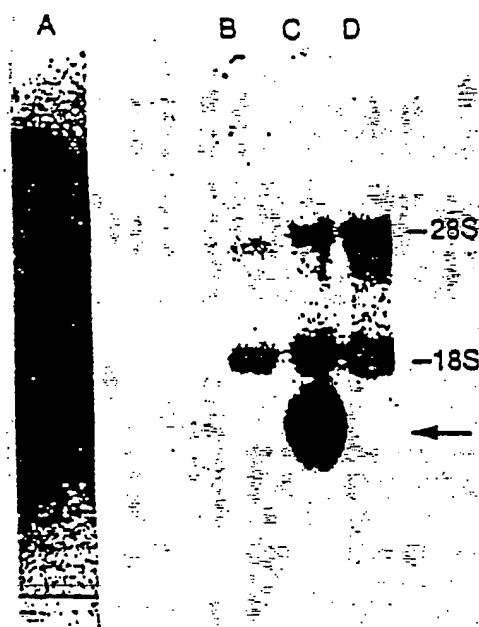


Fig. 6. Northern blot analysis of G418-selected pSV2NEOSVEB1a/MTONCO co-transfected primary BMK cells. The arrow by lane D indicates the position at which oncomodulin RNA migrates within a 1.0% agarose/formaldehyde gel. Of RNA extracted from three randomly selected cell lines (lanes B, C and D) producing polyomavirus-specific RNAs, at least one cell line (lane C) also produces oncomodulin-specific RNA from integrated MTONCO sequences.

BMK cells indicates that oncomodulin is not an immortalizing agent *in vitro*.

Discussion

As rat oncomodulin is expressed in a wide variety of tumors, it was of interest to determine if oncomodulin in any way contributes to or facilitates the development of cancer. In this initial study, it was investigated whether oncomodulin acts in a manner analogous to oncogenes *in vitro*. For this purpose, an expression vector containing the rat oncomodulin cDNA was introduced into both an established cell line and primary cells derived from mouse kidney.

A recombinant plasmid (pMTONCO), in which the synthesis of oncomodulin-specific RNA is under the direction of the metallothionein promoter, was used in these studies. The pMTONCO plasmid was introduced into Rat-1 cells and the expression of oncomodulin-specific RNA was verified. The two cell lines chosen for further study, MTONCO2-7 and MTONCO2-12, produced oncomodulin-specific RNA of different length. We believe this difference is due to differential utilization of polyadenylation signals, where MTONCO2-7 cell line uses the authentic oncomodulin polyadenylation signal,

and the MTONCO2-12 cell line utilizes the SV40 polyadenylation signal. It is not known why these different cell lines employ different polyadenylation signals, although it is clear that the SV40 signal is normally the preferred site, since four of the five oncomodulin RNA-producing cell lines utilize the SV40 polyadenylation signal (data not included). The pMTONCO plasmid did direct the synthesis of the oncomodulin protein, since it was shown that only in the MTONCO2-7 and MTONCO2-12 cell lines could a protein of predicted molecular weight be specifically immunoprecipitated with antiserum directed against oncomodulin. Since the level of oncomodulin protein, as judged by radioimmunoassay, was comparable to the level seen in transformed cell lines (MacManus *et al.* 1982), we presume that the MTONCO2-7 and MTONCO2-12 cell lines are suitable candidates for the analysis of oncomodulin gene function.

Two oncomodulin-producing cell lines, MTONCO2-7 and MTONCO2-12, were derived from Rat-1 cells, an established rat cell line. Both cell lines were compared with an oncogenically transformed cell line, since many of the phenotypic changes unique to transformed cells affect the rate and pattern of cell growth. The MTONCO2-7 and MTONCO2-12 cell lines showed similar density-dependent growth inhibition present in nontransformed cell lines (Holley and Kiernan, 1968). By contrast with normal cells, many transformed cell lines grow efficiently in semi-solid medium (MacPherson and Stoker, 1964). Oncomodulin-producing cell lines were unable to form colonies in soft agar, and displayed growth rates and saturation densities comparable to the untransformed parental cell line. Therefore, by a number of criteria, the presence of oncomodulin in an established cell line does not appear to confer growth characteristics normally associated with cellular transformation. We have not tested whether the MTONCO2-7 and MTONCO2-12 cell lines are able to form tumors in syngeneic animals. However, since a loose correlation exists between growth in soft agarose *in vitro* and tumor formation *in vivo* (Freedman and Shin, 1974; Shin *et al.* 1975), oncomodulin-producing cells would not be expected to be tumorigenic, although this possibility cannot be dismissed entirely.

To investigate whether oncomodulin might act in a more subtle manner to influence oncogenic transformation, the MTONCO2-7 and MTONCO2-12 cell lines were transformed with the pSV2SVEB1a plasmid. While oncomodulin is not able to initiate transformation of Rat-1 cells, it is conceivable that the presence of oncomodulin might make cells more susceptible to the effects of a transforming oncogene. From our results we conclude that, in this *in vitro* system, expression of oncomodulin does not increase the transforming frequency of the pSV2NEOSVEB1a plasmid, nor does it appear to affect the growth characteristics of polyomavirus-transformed cells.

Since the highest expression of oncomodulin is normally found in the outer placenta (Brewer and MacManus, 1985), it is tempting to speculate that oncomodulin might function to enhance tumor invasiveness or metastasis. This hypothesis seems unlikely, since

oncomodulin is also normally present in non-invasive amnion (Brewer and MacManus, 1985), and it has been shown that no correlation exists between the level of oncomodulin expression and tumor vigor (MacManus and Whitfield, 1983). Oncomodulin-producing polyoma-virus-transformed cells were not tested for their ability to form tumors in animals, although this analysis would directly address whether the expression of oncomodulin confers a growth advantage to transformed cells *in vivo* during tumor formation.

Some oncogenes, while not able to transform cells in culture, appear to be able to indefinitely prolong the lifespan of primary cells in cultures and have been referred to as immortalizing genes (Rassoulzadegan *et al.* 1982; Land *et al.* 1983; Ruley, 1983). Though we have demonstrated that oncomodulin-specific RNA can be produced by the pMTONCO construct in primary cells, we were unable to show that expression of oncomodulin altered the lifespan of primary cells in culture. Thus we conclude that oncomodulin does not act in a manner similar to immortalizing agents.

To date, our attempts to uncover an oncomodulin-specific activity that would play a role in tumor development have been unsuccessful. While the results presented here rely heavily on *in vitro* tissue culture systems, parallel experiments were initiated to assess the role of oncomodulin *in vivo*. For this purpose the pMTONCO plasmid was microinjected into fertilized mouse embryos in order to generate transgenic mice. The results of these experiments will be discussed extensively elsewhere (Chalifour *et al.* 1989). However, it appears that expression of oncomodulin is incompatible with the normal development of the mouse, and live births of oncomodulin-expressing transgenic animals were not observed. Though we can conclude that oncomodulin does not act like an oncogene *in vitro*, we cannot rule out the possibility that the expression of oncomodulin in some way influences tumor formation *in vivo*.

We thank J. A. Hassell, J. P. MacManus and B. Massie for providing some of the reagents used in these studies. We are grateful to J. P. MacManus for performing the radioimmunoassays. We also thank F. Gossard for helpful discussions and critical reading of the manuscript.

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Insulinotropin: Glucagon-like Peptide I (7-37) Co-encoded in the Glucagon Gene Is a Potent Stimulator of Insulin Release in the Perfused Rat Pancreas

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Abstract

Insulin secretion is controlled by a complex set of factors that include not only glucose but amino acids, catecholamines, and intestinal hormones. We report that a novel glucagon-like peptide, co-encoded with glucagon in the glucagon gene is a potent insulinotropic factor. The glucagon gene encodes a proglucagon that contains in its sequence glucagon and additional glucagon-like peptides (GLPs). These GLPs are liberated from proglucagon in both the pancreas and intestines. GLP-I exists in at least two forms: 37 amino acids GLP-I(1-37), and 31 amino acids, GLP-I(7-37). We studied the effects of synthetic GLP-I on insulin secretion in the isolated perfused rat pancreas. In the presence of 6.6 mM glucose, GLP-I(7-37) is a potent stimulator of insulin secretion at concentrations as low as 5×10^{-11} M (3- to 10-fold increases over basal). GLP-I(1-37) had no effect on insulin secretion even at concentrations as high as 5×10^{-7} M. The earlier demonstration of specific liberation of GLP-I(7-37) in the intestine and pancreas, and the magnitude of the insulinotropic effect at such low concentrations, suggest that GLP-I(7-37) participates in the physiological regulation of insulin secretion.

Introduction

Pancreatic glucagon and intestinal glicentin are synthesized in the form of a 180-residue protein, proglucagon encoded in a single gene (1). The precursor contains in addition to glicentin and glucagon the sequences of two glucagon-like peptides (GLPs)¹, GLP-I and GLP-II, separated by an intervening peptide (IP-II) (2-5). The posttranslational processing of proglucagon differs in pancreas and intestine (1, 6). In the pancreas the precursor is processed to glucagon and GLP-I, and in both large and small intestines glicentin, GLP-I, GLP-II and IP-II-leucine-amide are found. Both pancreas and intestine contain GLP-I in at least two forms—31 and 37 residues long (1).

The close similarity of the amino acid sequence of GLP-I and GLP-II with glucagon and the other peptides related in structure to glucagon (secretin, vasoactive intestinal peptide, gastric inhibitory peptide, growth hormone-releasing hormone)

suggests that the GLPs might have a role in metabolic regulation. The specific liberation of GLP-I and GLP-II in the intestine indicates that these peptides may be components of the entero-insular axis (7), which comprises multiple intestinal factors influencing the release of hormones produced in the pancreatic islets. Further, they may be incretins, endocrine transmitters produced in the gastrointestinal tract that are released by nutrients and stimulate insulin secretion in the presence of elevated glucose if exogenously infused in amounts not exceeding blood levels achieved after food ingestion (8). Detection of both GLP-I(1-37) and GLP-I(7-37) in pancreas and intestines raises the possibility that GLP-I(1-37) is itself a prohormone that undergoes a proteolytic cleavage at the single arginine residue at position 6 to release the biologically active GLP-I(7-37). In these studies we used synthetic GLP-I(1-37) and GLP-I(7-37) to investigate their effects on insulin secretion in the perfused rat pancreas and find that GLP-I(7-37) has uniquely potent insulinotropic actions.

Methods

Synthesis of peptides. Glucagon and GLP-I were synthesized by the stepwise solid-phase method (9). Because the assembly of the peptide chain proceeds in the carboxyl- to the amino-terminal direction, GLP-I(1-37) and GLP-I(7-37) were prepared in the same synthesis by separating the peptide resin after incorporation of a protected histidyl residue at position 7 and continuing the assembly of amino acids on the other aliquot of the peptide resin to obtain protected GLP-I(1-37) peptide resin. Peptides were purified by preparative reverse-phase C-18 chromatography. Purified peptides were shown to be homogeneous by amino acid analysis, preview-sequence analysis, and high performance liquid chromatography (HPLC) on reverse-phase C-18 and ion-exchange DEAE-S2 columns.

Radioimmunoassays. Development of the antisera and competitive binding radioimmunoassays for glucagon and GLP-I are described elsewhere (1). In brief, samples were incubated with the antisera in borate buffer (pH 8.1) for 24 h at 0°C, followed by addition of ¹²⁵I-labeled peptide for an additional 24 h in a total volume of 0.5 ml. Separation of the antibody bound from the free peptide was accomplished with dextran-coated charcoal. Assay sensitivity with all three antisera was 10 pg/ml. The antiserum against GLP-I was obtained by immunization with GLP-I(1-37) and is directed against both the amino-terminal (1-6) part of the molecule and to 7-37 determinants. Therefore, the amount of GLP-I(7-37) may be over or underestimated with respect to GLP-I(1-37) in the assay. The assay for insulin (10) used charcoal separation and rat insulin standards (Novo Research Institute, Copenhagen, Denmark).

Rat-perfused pancreas experiments. The preparation of the in situ isolated rat pancreas has been described previously (11, 12). The perfusate contained bicarbonate buffer (pH 7.4) and 120 mg/dl glucose, 4% dextran T-70, and 0.2% bovine serum albumin, and was equilibrated with 95% O₂ and 5% CO₂. The first 20 min of each perfusion was an equilibration period and is not represented in the data graphs.

Received for publication 23 October 1986.

1. Abbreviation used in this paper: GLP, glucagon-like peptide.

J. Clin. Invest.

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0021-9738/87/02/0616/04 \$1.00

Volume 79, February 1987, 616-619

After the initial 20-min equilibration period, aliquots of perfusate were removed every 2-4 min for additional 20 min, thus allowing the system to equilibrate for a total of 40 min. The perfusion, with GLP-I(1-37) or GLP-I(7-37), was for 6 min and samples were collected at 1-min intervals. The peptide perfusions were followed by equilibration periods of 20 min, during which four samples 5 min apart were collected. A second 6-min perfusion followed with the same peptide as the first perfusion only at 100 times higher concentration of peptide. Again, samples 1 min apart were collected. The entire perfusion time was between 70 and 85 min.

In each aliquot of perfusate obtained, insulin was determined by radioimmunoassay. In addition the efficiency of delivery of the GLP-is was confirmed by radioimmunoassay of corresponding aliquots of perfusate in which insulin was measured (1).

Results

To optimally study the effects of GLP-I(7-37) and GLP-I(1-37) on insulin secretion we used separate perfusions with each peptide, perfusing twice at two different concentrations of peptides and allowing 20-min time intervals between the two perfusions. In perfusions of two separate pancreases using this protocol, GLP-I(7-37) was a potent stimulator of insulin secretion, giving about a 20-fold stimulation at 5×10^{-11} M and a sixfold stimulation at 5×10^{-12} M (Fig. 1). In comparison, GLP-I(1-37), also studied in two pancreases, showed no effect on insulin secretion at either 5×10^{-9} or 5×10^{-7} M (Fig. 2). At the latter concentration no effect was observed even during a 15-min perfusion period (Fig. 2B).

Using a slightly different perfusion protocol than that described above (Figs. 1 and 2) we gave alternate 5-min infusions of the peptides at concentrations ranging from 5×10^{-7} to 5×10^{-12} M to five additional individual pancreases. We reproducibly observed insulin release in response to GLP-I(7-37) at concentrations as low as 5×10^{-11} M, and little if any insulin responses to GLP-I(1-37) at concentrations as high as 5×10^{-7} M. Thus, the potent insulinotropic actions of GLP-I(7-37) have been observed in studies of seven separate pancreases.

Effects of glucagon on insulin secretion in the perfused pancreases have been established previously (13). We also compared the effects of glucagon to that of the GLP-is. We used synthetic

glucagon in the concentration range of 10^{-11} - 10^{-7} M and found it to be less potent than GLP-I(7-37).

Discussion

The results of these studies clearly indicate that GLP-I(7-37) has potent insulinotropic activity. The liberation of this peptide from proglucagon in the intestine, and to a lesser extent in the pancreas (1), raises the possibility that GLP-I(7-37) has a role in endocrine regulation in the entero-insular axis (7). Our data, taken together with earlier observations that glucagon-like immunoreactivity in crude gut extracts released insulin after ingestion of glucose and fat (8) suggest that GLP-I(7-37) could potentially be an incretin. Of all the known intestinal hormones tested for their insulin-releasing potency in the past, gastric inhibitory peptide has been considered as a possible incretin (14, 15). However, the concentrations of gastric inhibitory peptide required to stimulate insulin secretion exceed the physiologic levels of the peptide achieved after a meal. In the rat-perfused pancreas in the presence of 8.9 mM glucose, gastric inhibitory peptide (10^{-6} M) increased insulin secretion sixfold (16). We find a comparable increase in insulin secretion with GLP-I(7-37) at concentrations 100-fold lower than those required for an insulinotropic response to gastric inhibitory peptide. By radioimmunoassay we have measured both GLP-I(1-37) and GLP-I(7-37) levels of ~ 150 pg/ml (50 pM) in rat portal blood and 50 pg/ml (15 pM) in peripheral blood (S. Mojsov, unpublished results). Therefore, the insulinotropic effect that we have observed at concentrations of GLP-I(7-37) of between 5 and 50 pM are well within the physiological levels of GLP-I(7-37) found in the circulation.

There has been considerable interest in the potential intra-islet relationships which might occur between A, B, and D cells, such that the secretory product of one cell type might influence the function of a neighboring cell (17). Interaction could take place via a paracrine mechanism or through a local intra-islet portal system. Glucagon can stimulate both insulin and somatostatin secretion (12, 18), but because there appears to be a functional compartmentalization between islet cells, it is unclear whether glucagon can actually reach B and D cells (19). Taking into account the vascular arrangement of the rat islet, the glu-

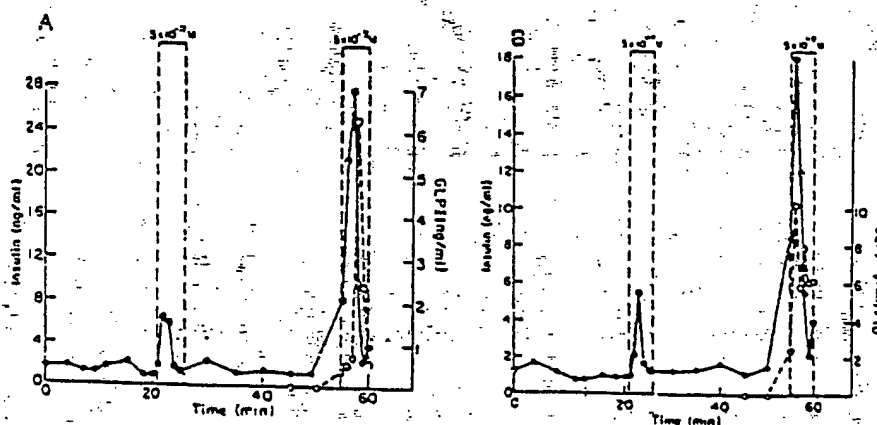


Figure 1. The effects of separate perfusions in two representative pancreases GLP-I(7-37) at two concentrations, 5×10^{-11} and 5×10^{-12} M. Solid lines, insulin values determined by radioimmunoassay. Dashed lines, amount of peptide perfused as determined in a competitive binding radioimmunoassay with antisera against GLP-I(1-37). The amount of GLP-I(7-37) at 5×10^{-11} M is beyond the detection sensitivity of the radioimmunoassay. Each graph represents a perfusion of a separate pancreas with a given peptide.

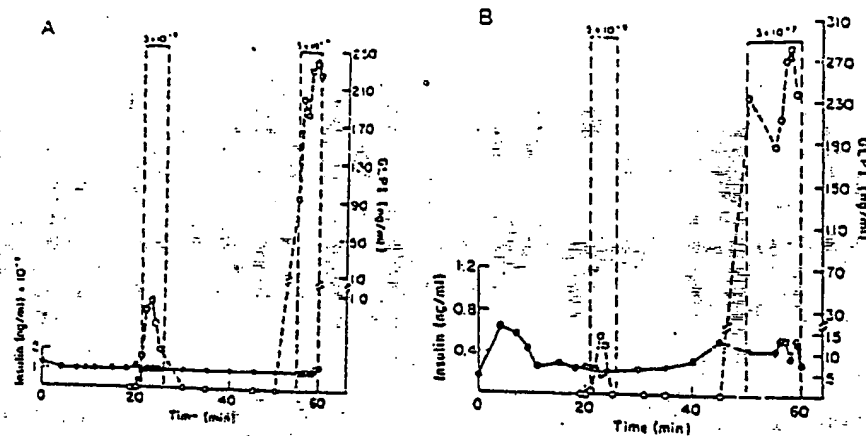


Figure 2. The effects of separate perfusions in two representative pancreas with GLP-I(1-37) at two concentrations, 5×10^{-11} and 5×10^{-10} M. Details of the experiment and explanation of symbols are described in legend to Fig. 1.

agon-containing A cells of the mantle appear to be downstream from the B cells of the core, and therefore glucagon may not reach the B cells in high enough concentration to exert a significant influence (20). The mantle of A and D cells are, however, adjacent and this makes the possibility of paracrine interaction more feasible, although experimental support for such an interaction is not available. The finding that GLP-I(7-37) is a more potent insulin secretagogue than glucagon raises important questions about its potential intra-islet role.

Amino and carboxyl-termini of glucagon, GLP-I(7-37) and GLP-II are closely related to each other in their amino acid sequences and to vasointestinal peptide that possibly exerts a neuronal stimulation of insulin secretion (21). A most striking similarity among them is the conservation of a histidine residue at position 1. It is noteworthy that gastric inhibitory peptide, also closely related in its structure to the GLPs, has a tyrosine residue at position 1 instead of histidine (22). Inasmuch as a histidine residue at this position is essential for adenylate cyclase stimulation in various systems, the greater insulinotropic potency of GLP-I(7-37) compared with GIP may in part be accounted for by the histidine substitution for tyrosine (23).

Additional evidence in support of the concept that GLP-I(7-37) is a potent insulinotropic peptide is provided by our recent observation that GLP-I(7-37), and not GLP-I(1-37) or GLP-II, is a potent activator of adenylate cyclase at concentrations as low as 5×10^{-11} M and also stimulates cellular levels of insulin mRNA and insulin release in a rat insulinoma cell line (RIN-38) (Drucker, D. J., J. Philippe, S. Mojsov, W. L. Chick, and J. F. Habener, manuscript in preparation). Further, studies by Schmidt and co-workers showed that in isolated precultured islets, 10^{-9} to 10^{-6} M concentrations of the peptide GLP-I(1-36 des Gly-Arg amide) were required to release insulin (24).

Determining whether GLP-I(7-37) is the hormone whose primary function is to stimulate insulin secretion in response to feeding, or is one of a complex group of hormones involved in maintaining glucose homeostasis, will require further investigation.

Acknowledgments

We thank Henrietta Cooper, Deanna Deery, and Adacie Allen for their expert experimental assistance in carrying out the pancreatic perfusions

and radioimmunoassay, respectively. We thank Esther Hoomis for typing the manuscript.

The studies were supported in part by Public Health Service grants AM 30824 and AM 20349.

Note added in proof. In studies of isolated perfused pig ileum and pancreas, Orskov et al. (25) recently reported finding secretion of a GLP-I peptide from ileum, but the pancreas secreted a large peptide with both GLP-I and GLP-2 immunoreactivity.

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